AD_____

Award Number: DAMD17-97-1-7208

TITLE: Role of the ATM Gene in Bilateral Breast Cancer Following Radiotherapy

PRINCIPAL INVESTIGATOR: Doctor Barry Rosenstein

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029-6574

REPORT DATE: August 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Final (11 Jul 97 - 10 Jul 01)

4. TITLE AND SUBTITLE
Role of the ATM Gene in Bilateral Breast Cancer Following Radiotherapy

5. FUNDING NUMBERS
DAMD17-97-1-7208

6. AUTHOR(S)
Doctor Barry Rosenstein

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai School of Medicine
New York, New York 10029-6574

E-Mail: barry.rosenstein@mssm.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this project was to screen women who developed either unilateral or bilateral breast cancer for mutations and polymorphisms in the *ATM* gene. To accomplish this, during the first three years of this project, DNA samples were isolated from breast cancer patients and screened using a *N*on-radio*i*sotopic *R*Nase *C*leavage-based *A*ssay (NIRCA). However, not a single mutation was discovered from any of these patients which raised a concern as to the sensitivity of this assay. To address this concern, the PI of this project purchased an instrument for the performance of denaturing high performance liquid chromatography (DHPLC). This is a high throughput technique in which large numbers of DNA samples can be rapidly screened with a high degree of sensitivity and accuracy. During the fourth year of this study, representing a no-cost extension of this project, it was possible to re-screen all of the DNA samples obtained from the unilateral breast cancer patients for *ATM* mutations using DHPLC. Through this work, we identified 6 breast cancer patients possessing a total of 8 different germline mutations and 43 patients harboring a total of 141 DNA sequence variations representing 21 different common polymorphisms and rare variants.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
97

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Ataxia telangiectasia (AT), which results from mutations in both copies of the *ATM* gene, is relatively rare with an incidence of approximately only 1 in 100,000. However, the heterozygous condition which is characterized by a mutation in one copy of the gene, is thought to affect roughly 0.5-1% of the population. These individuals do not suffer from AT, however, they appear to be particularly susceptible to the induction of breast cancer by radiation. To examine this hypothesis, breast cancer patients were screened for the presence of mutations and polymorphisms in the *ATM* gene.

BODY

Task 1: Month 1: Identify with Dr. Paul Tartter the bilateral breast cancer patients who fall into the two categories to be used in this study.

This was accomplished using records Dr. Tartter has assembled for bilateral breast cancer patients treated at Mount Sinai over the past 18 years.

Task 2: Month 1: Establish a procedure by which the breast cancer patients will be recruited into the study.

There was relatively easy accessibility to unilateral breast cancer patients as many of these patients were treated in the Radiation Oncology Department and continue to come to Mount Sinai periodically for follow-up visits. Arrangements were made so that when patients selected for this study arrived for their exam, an investigator was available to explain the project to the patients and obtain informed consent for participation in the study following which a nurse would draw a blood sample. A difficulty arose, however, recruiting bilateral breast cancer patients into the study as many of these patients, who were treated as long as 18 years ago, had either died, been lost to follow up or rarely visited Mount Sinai. Therefore, a alternate strategy was developed to access the bilateral patients in which paraffin embedded tissue, preferably lymph node biopsy samples, were obtained for these individuals. A collaboration was established with Dr. Ira Bleiweiss, the pathologist at Mount Sinai responsible for review of all breast cancers. Using this approach, we successfully retrieved paraffin-embedded tissue blocks from 41bilateral patients identified by Dr. Tartter.

Task 3: Month 1: Decide which regions of ATM will be subjected to analysis based upon results available in the literature at the time the project begins

As of this point, there are no specific allelic hotspots or regions of *ATM* which have been identified for mutations. Therefore, the entire coding region of the gene is being examined.

Task 4: Months 2-32: Obtain blood samples from breast cancer patients

Blood samples were obtained from a total of 37 unilateral breast cancer patients. In addition, tissue blocks were retrieved for 41 bilateral breast cancer patients.

Task 5: Month 2: Create appropriate primers for RT-PCR and sequencing

During the period when a procedure to recruit bilateral breast cancer patients into the study was being established, efforts were also initiated to create primers for RT-PCR. As this effort was well underway by the time a decision was made to use genomic DNA obtained from paraffin embedded tissue, it was decided to complete construction of the cDNA primers and use them to screen for *ATM* mutations in unilateral breast cancer patients. The results of this portion of the project have been published and a reprint of this manuscript (Drumea et al, 2000) has been included in the appendix to this report. In addition, primers were designed and validated for use with genomic DNA to amplify all of the exons that comprise the *ATM* gene.

Task 6: Months 3-36: Isolate mRNA from blood lymphocytes of patients

As described, rather than isolating mRNA from blood samples, genomic DNA was isolated from paraffin-embedded biopsy tissues. To accomplish this, the ONCOR EX-WAX™ DNA Extraction Kit was used. The procedure employed was to first cut 10 sections of 5 μ thickness from a paraffin-embedded lymph node biopsy sample and place them in a 1.5 ml tube. 1 ml of 100% ethanol was added, vortexed 15 sec, centrifuged at 12,000 rpm 3 min, the ethanol removed and the pellet air dried. 150 μl of digestion solution and 50 μl of protein digesting enzyme solution were added and the tube incubated 4-18 hr at 50°. 100 μl of extraction solution was mixed and after 15 sec the tube was centrifuged 10 min at 12,000 rpm. The supernatant was removed by poking the pipette tip through the paraffin layer on top and withdrawing the supernatant, leaving the paraffin and pellet behind. 150 μl of precipitation solution was added to the supernatant, the tube inverted 3 times, 900 μl of 100% ethanol (-20°) added and mixed. After 1 hr the

tube was centrifuged for 10 min at 12,000 and the supernatant discarded. The pellet was air dried and 50 µl of resuspension solution added for 1 hr at 50°.

Task 7: Months 3-36: Perform RT-PCR with these samples to amplify in sections the coding region of the *ATM* gene.

RT-PCR was not necessary as genomic DNA samples were used. The first stage PCR reactions were performed using approximately 100-500 ng genomic DNA, 125 µM dNTPs, 250 nM primers, 1 U Taq polymerase in a buffer containing 10 mM Tris.Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin and RNase free water to a total volume of 50 µl. The PCR reactions were performed in a Perkin Elmer 9700 Thermal Cycle at 94° for 4 min, followed by 30 cycles of 1 min at 94°, 2 min at 54° and 1.5 min at 72° and one cycle for 8 min at 72°. 10 µl of the first stage PCR product was used in a second series of PCR reactions using the same conditions to achieve further amplification of the region of interest and to add the T7 and SP6 promoters to the PCR products.

Task 8: Months 3-36: Use NIRCA to identify PCR products containing mutations

All *ATM* exons were amplified using PCR for the DNA samples isolated from the bilateral breast cancer patients and screened for mutations using a Non-radioisotopic RNase Cleavage-based Assay (NIRCA). Each second stage PCR product was transcribed in two reaction mixes using 2 µl of the PCR product, 250 nM rNTPs, 2 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris.Cl, pH 7.5, 7 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 10 µl with RNase free H_2O and incubated for 1 hr at 37°. 10 µl of buffer containing 80% (v/v) formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0 was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95° for 3 min and allowed to cool at room temperature for 1 min. 4 µl of the duplex reactions were mixed with 16 µl of RNase digestion buffer (10 mM NaCl, 10 mM Tris.Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with 0.5 µg/ml RNase A, RNase I and RNase T1 and brought to a final volume of 20 µl. The reaction mixtures were incubated at 37° for 20 min and resolved on a 3% agarose gel run at 100 V for approximately 45 min.

Task 9:Months 3-36: Sequence all PCR products which appear to exhibit mutations

No mutations were identified in any of the DNA samples isolated from either the unilateral or bilateral breast cancer patients and therefore it was not necessary to perform DNA sequencing. However, the inability to detect even a single mutation

in this study raised a concern that the assay used for this work, NIRCA, did not possess sufficient sensitivity to detect the small number of mutations that are likely to be present in this breast cancer population. To address this concern, the PI of this project recently obtained a denaturing high performance liquid chromatography (DHPLC) system from Transgenomic. This purchase was accomplished from internal Mount Sinai funding sources.

Through use of DHPLC, single-base substitutions, small insertions and deletions can be detected in 100-1500 bp DNA fragments by fractionation of heteroduplexes on ion-pair reverse-phase columns. The Wave™ DNA Fragment Analysis System manufactured by Transgenomic, that has been installed in the PI's laboratory and is available for this project, is a complete unit for the automated DHPLC analysis of PCR products in a 96 well plate using a DNASep cartridge specifically designed for separation of DNA fragments. This represents a high throughput technique in which large numbers of DNA samples can be rapidly screened for base sequence alterations.

DHPLC relies upon the physical changes in DNA molecules induced by mismatched heteroduplex formation during reannealing of wild type and mutant DNA. In this method, a portion of a gene is amplified using standard PCR conditions and the products analyzed using DHPLC. Material from a homozygous sample will only form one species, the wild-type homoduplex, which will appear as a single peak on the DHPLC chromatogram. However, when the PCR products produced from a sample heterozygous for a base sequence alteration are heated to 95°C, and then slowly cooled, the DNA strands separate and randomly reanneal to form a mixture of three species; a mutant homoduplex, a heteroduplex and a wild type homoduplex. These will appear ideally as four peaks on a chromatogram, although often only two or three peaks are present as either the two homoduplexes or two heteroduplexes may not be resolvable from each other.

On 6/29/00, Ms. Cheryl Miles was informed that we wished to exercise our option for a one-year no-cost extension of this grant so that the new termination date for this project is 8/10/01. The purpose of this extension was to reanalyze all of the samples obtained from the breast cancer patients using the rapid and sensitive DHPLC instrument to determine whether any mutations went undetected using NIRCA. Over the past year we were able to accomplish the re-screening of all 37 unilateral breast cancer patients plus an additional 15 patients. The results of this work are summarized in three papers that are included in the Appendix (Atencio et al., in press; Atencio et al., submitted for publication; Iannuzzi et al., submitted for publication). Unfortunately, due to limitations in time and funding, it was not possible to screen the bilateral breast cancer patients for *ATM* mutations.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of 6 breast cancer patients possessing a total of 8 different novel germline *ATM* mutations and 43 patients exhibiting a total of 141 DNA sequence variations representing 21 different common polymorphisms and rare variants.

- Demonstration that the incidence of *ATM* heterozygosity in the sample of breast cancer patients studied (12%) was significantly ($p < .05$) greater than the 1% of the general population thought to be carriers of a mutation in the *ATM* gene. This result is consistent with the conclusion that women who are carriers of a mutation in the ATM gene are at increased risk for the development of a breast cancer.

- Demonstration of a significant difference in the incidence of *ATM* mutations among African-American breast cancer patients (3/7 = 43%) compared with other ethnic groups (3/45 = 7%).

- Discovery of the first person to have been identified possessing two mutated copies of the *ATM* gene, but not exhibiting classical AT-associated characteristics.

- Demonstration that 100% (3/3) of the breast cancer patients, treated with radiotherapy and developing grade 3-4 subcutaneous late sequelae, possessed *ATM* mutations. In contrast, only 7% (3/43) of the patients who did not manifest this form of severe toxicity harbored an *ATM* mutation. This finding suggests that all breast cancer patients should be screened for *ATM* mutations as possession of an altered *ATM* gene appears to be predictive of an increase in subcutaneous late effects following treatment with radiation. Therefore, possession of an *ATM* mutation may prove to be a relative contraindication to standard management with radiotherapy.

REPORTABLE OUTCOMES

Drumea, Karen C., Eva Levine, Jonine Bernstein, Brenda Shank, Sheryl Green, Lynda Mandell, Joan Cropley, Juliette Obropta, Irene Braccia, Amy Krupnik and Barry S. Rosenstein. *ATM* Heterozygosity and Breast Cancer: Screening of 37 Breast Cancer Patients for *ATM* Mutations Using a Non-Isotopic RNase Cleavage-Based Assay. *Breast Cancer Research and Treatment* 61:79-85, 2000.

Atencio, David P., Christopher M. Iannuzzi, Sheryl Green, Richard G. Stock, Jonine L. Bernstein and Barry S. Rosenstein. Screening breast cancer patients for *ATM* mutations and polymorphisms using denaturing high performance liquid chromatography. *Environmental and Molecular Mutagenesis* (in press)

Atencio, David P., Chritopher M. Iannuzzi, Kevin Hopkins, Jonine L. Bernstein, Sheryl Green, Howard B. Lieberman and Barry S. Rosenstein. Identification of a breast cancer patient possessing two mutated copies of the *ATM* gene but no symptoms of ataxia telangiectasia. *American Journal of Human Genetics* (submitted for publication)

Iannuzzi, Christopher M., David P. Atencio, Sheryl Green, Richard G. Stock and Barry S. Rosenstein. *ATM* heterozygosity in female breast cancer patients predicts for an increase in radiation-induced late effects. *International Journal of Radiation Oncology Biology Physics* (submitted for publication)

CONCLUSIONS

Using DHPLC, we have discovered that breast cancer patients have a higher incidence of *ATM* heterozygosity than the general population and therefore possession of an *ATM* mutation may render a woman susceptible to the development of breast cancer. This may be particularly true for African-American women. In addition, being a carrier of *ATM* mutations results in a greater chance that a breast cancer patient undergoing radiotherapy will develop complications resulting from treatment. Finally, we have identified the first person to possess two mutated copies of the *ATM* gene, but not to exhibit the disease ataxia telangiectasia. This demonstrates that possession of two missense mutations do not necessarily cause AT, although such a person may be more susceptible to breast caner formation.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Barry S. Rosenstein
Jonine L. Bernstein
David P. Atencio

Breast Cancer Research and Treatment **61:** 79–85, 2000.

ATM heterozygosity and breast cancer: screening of 37 breast cancer patients for *ATM* mutations using a non-isotopic RNase cleavage-based assay *

Karen C. Drumea[1,4], Eva Levine[1], Jonine Bernstein[2], Brenda Shank[1], Sheryl Green[1], Edward Kaplan[5], Lynda Mandell[1], Joan Cropley[1], Juliette Obropta[1], Irene Braccia[1], Amy Krupnik[1], and Barry S. Rosenstein[1,2,3]

[1]*Department of Radiation Oncology,* [2]*Department of Community Medicine & Preventive Health,* [3]*Department of Dermatology, Mount Sinai School of Medicine of New York University, New York, USA;* [4]*Department of Oncology, Rambam Medical Center, Haifa, Israel;* [5]*Boca Raton Radiation Therapy Center, Dearfield Beach, USA*

Key words: ataxia telangiectasia, *ATM*, breast cancer, mutation screening

Summary

Based upon the results of several epidemiologic studies, it has been suggested that women who are carriers for a mutation in the ataxia telangiectasia-mutated (*ATM*) gene are susceptible for the development of breast cancer. Therefore, 37 consecutive breast cancer patients were screened for the presence of a germline *ATM* mutation using a non-isotopic RNase cleavage-based assay (NIRCA). This paper reports the first use of NIRCA for detection of *ATM* mutations in breast cancer patients. Using this assay, no *ATM* mutations were found in our patient population. This result is similar to the findings of other studies that have employed approaches complementary to NIRCA.

Introduction

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar degeneration with ataxia, conjunctival telangiectasias, immunodeficiency, chromosomal instability, radiation sensitivity and cancer predisposition [1]. The cancer risk exhibited by AT patients is believed to be approximately 60–180 times higher than that of the general population [2], consisting primarily of lymphomas and leukemias. Ataxia telangiectasia-mutated (*ATM*), the gene whose alteration results in AT, has been cloned and mapped to chromosome 11 q22-23 [3, 4]. *ATM*, which is expressed in a wide variety of tissues, is approximately 150 kb in length possessing 66 exons with a 12 kb transcript. The open reading frame of 9168 nucleotides encodes a nuclear phosphoprotein of 3056 amino acids. Most AT patients are compound heterozygotes with the location of mutations spread throughout the genome [5]. Concannon and Gatti [6] found that 72% of 78 unique mutations demonstrated in AT patients would be predicted to produce either a truncated protein or no product.

A hallmark of this disease is great sensitivity to radiation as AT patients who were exposed to conventional radiation doses for cancer treatment developed devastating tissue necrosis [7]. The basis for this radiosensitivity has long been the subject of investigation that advanced significantly with cloning of the *ATM* gene. It has been demonstrated that the *ATM* protein belongs to an expanding family of large eukaryotic proteins involved in intracellular signaling, cell cycle control, DNA repair and recombination in response to DNA damage. This family of proteins has been grouped together with *ATM* primarily based upon the strong homology of their carboxyl termini to the 100 kDa catalytic subunit of the mammalian signal transduction mediator phosphatidylinositol-3 (PI-3) kinase. PI-3 kinases appear to participate in many

* This paper is dedicated to the memory of Eva Levine, whose extraordinary sensitivity and devotion to her patients was an inspiration to her colleagues and will forever he missed.

cellular processes, including insulin-dependent glucose transport, growth factor responses and cellular differentiation [8–10].

The worldwide incidence of AT is estimated at 1:40,000–1:100,000 live births [11, 12]. In contrast to the relative rarity of this disease resulting from *ATM* homozygosity, heterozygosity is far more common and it has been estimated that approximately 1% of the population possesses a mutation in one copy of this gene [13]. These heterozygotes do not manifest any outward radiation sensitivity, but, cells derived from individuals who are carriers for a mutation in this gene exhibit a slightly increased radiation sensitivity [14, 15].

Based upon the results of epidemiologic studies, it has been suggested that *ATM* carriers have a 2–6 fold greater risk for cancer development and are particularly predisposed to breast cancer compared with non-carriers [16–18]. It has been estimated that 9–18% of all women with breast cancer may be *ATM* heterozygotes [19]. To address this issue, Easton [20] performed a pooled analysis of four studies [16, 17, 19, 21] from which it can be suggested that AT heterozygotes are more prone to breast cancer and could account for up to 13% of breast cancer cases, with 3.8% being the best estimate. However, recent studies have cast a doubt upon this suggestion as the level of mutations detected among breast cancer patients has been consistent with only the 1% frequency estimated for the general population [22–24]. In addition, *ATM* mutations have not been detected in breast cancer patients who developed unusually severe responses to radiotherapy indicating that *ATM* heterozygosity does not result in acute complications associated with this treatment [25–27].

All of the studies performed to date have been accomplished using either the protein truncation test (PTT), restriction-endonuclease fingerprinting (REF) or single strand conformation polymorphism (SSCP). However, it is important to note that none of these techniques is capable of detecting all mutations and it is always essential to use a series of screening methods to locate most mutations in a population. It is, therefore, important to employ a complementary assay with a different basis to identify mutations which had possibly gone undetected using other techniques. NIRCA represents such an alternative approach. Hence, the purpose of this study was to determine the prevalence of germlme *ATM* mutations in breast cancer patients using NIRCA.

Methods

Patients

Three milliliter of peripheral venous blood samples were obtained from a hospital-based series of 37 unselected patients diagnosed with primary breast cancer treated with radiation therapy with or without chemotherapy between 1992 and 1997. The nature of the project was discussed with each patient and signed informed consent was obtained prior to obtaining each blood sample. Patient characteristics were obtained by chart review and are presented in Table 1.

Table 1. Breast cancer patient characteristics

Patient characteristics	Number of patients (%)
Age Group (years)	
Under 40	2 (5.5)
40–59	14(37.8)
60–80	20 (54)
Over 80	1(2.7)
Median Age = 62	
Ethnic group	
Caucasian[a]	20 (54)
Hispanic	13 (35.2)
Black	3(8.1)
Asian	1(2.7)
Menopausal status	
Pre- and peri-menopausal	9 (24.3)
Postmenopausal	28 (75.7)
Family history of cancer	
Breast[b]	13 (35.2)
Other site	9 (24.3)
None	15 (40.5)
Stage of disease	
Stage 0	3 (8.1)
Stage I	27 (80)
Stage II	6 (16.2)
Stage III	1 (2.7)
Histology	
Intraductal	3 (8.1)
Infiltrating duct	26 (70.1)
Infiltrating lobular	2 (5.5)
Tubular	1 (2.7)
Tubulo-lobular	3 (8.1)
Adenocarcinoma	2 (5.5)

[a] 11 patients of Ashkenazi Jewish background.
[b] 7 patients with first-degree relatives.

RNA extraction and cDNA synthesis

Lymphocytes were isolated by Ficol-plaque separation. 1.5 ml Ficol was added to 3 ml of blood and centrifuged at 12000 rpm for 20 min at room temperature. The lymphocytes layer was collected, washed with 10 ml PBS and centrifuged for 10 min, room temperature, at 12,000 rpm. To isolate RNA from the lymphocytes, 2 ml RNAzol^{TM} (Cinna/TEL-TEST) was added to the cells, followed by addition of 100 μl of chloroform and centrifuged at 4°C for 15 min at 12,000 rpm. The supernatant was transferred to a 1.5 ml tube and an equal volume of isopropanol added to the supernatant. The tube was held at −20°C overnight and centrifuged 15 min at 4°C. The supernatant was removed and the pellet washed with 500 μl of 70% ethanol. After centrifugation at 4°C, 12,000 rpm, for 8 min, the supernatant was removed and the pellet washed with 200 μl of 70% ethanol and centrifuged once more at 4°C, 12,000 rpm speed, for 8 min. The supernatant was removed, the pellet air-dried and 30 μl RNase free H_2O added.

cDNA was synthesized using the isolated RNA as a template by combining 20 μl RNA solution with 4 μl random primers. The mixture was heated to 70°C for 10 min and quickly chilled on ice. After a brief centrifugation, 4 μl of 5 × first strand buffer, 2 μl 0.1 M DTT and 1 μl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at pH 7.0) were added and the mixture incubated 2 min at 42°C. Two micro litre (200 units) SUPERSCRIPT^{TM} was added, the mixture incubated 50 min at 42°C and then 70°C for 15 min. To remove RNA complementary to the cDNA, the reaction was treated with 2 units *E. coli* RNase H and incubated at 37°C for 20 min.

PCR conditions and primers

Segments of the *ATM* gene were amplified from aliquots of the cDNA template using sets of primer pairs. The primers were designed with the aid of Oligo-5 software (NBI, Plymouth, MN) and are listed in Table 2 (first stage primers) and Table 3 (second stage primers). The *ATM* open reading frame was amplified in a series of 12 overlapping fragments that were approximately 1 kb in size. The product of each PCR reaction was then used as a template for a second reaction to further amplify this region of the gene. In addition, the primers were designed so that sequences for the *T7* and *SP6* promoters were incorporated into the 5′ end of each sense and antisense primer, respectively. Two microlitre of cDNA was used in a 50 μl PCR reaction using 250 nM first stage primers, 125 μM dNTPs, 1 U Taq polymerase in a buffer containing 10 mM Tris-Cl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin. The PCR reactions were performed in a Perkin Elmer 9700 ThermalCycler with the following settings: 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and one cycle for 7 min at 72°C. One microlitre of first stage PCR product was used in the nested PCR reactions using the same conditions, with second stage primers.

Table 2. Sequences of first stage *ATM* primers

Fragment number	Bases amplified	Primer sequence
1-Sense	5–906	GAGGAGTCGGGATCTGC
1 -Antisense		GACAGCCAAAGTCTTGAG
2-Sense	695–1676	CTGTGTACTTCAGGCTCT
2-Antisense		GCTTGTATTTGCTCAGAA
3-Sense	1544–2509	CACCATATGTGTTACGAT
3-Antisense		TCATATTTCTCAAGGAAC
4-Sense	2329–3298	ACTCTTGTCCGGTGTTC
4-Antisense		AGGGCCATTCTTACAGA
5-Sense	2978–3977	TAATTGATTCTAGCACGC
5-Antisense		CTTCTAATCACCAGATGT
6-Sense	3877–4850	AACTTATCTTCTTTTCC
6-Antisense		AATCGTGATATAGAGGT
7-Sense	4710–5682	TTACTGTAAGGATGCTC
7-Antisense		AGTCAGTTTTCACTTCA
8-Sense	5559–6534	TGATATAAATCTGTGGA
8-Antisense		TTCTACTTCTTTGCTG
9-Sense	6407–7353	TCTGCCATATTCTTTCC
9-Antisense		AACCGGGCTAATGAG
10-Sense	7193–8172	CAGAATGTCTGAGGGT
10-Antisense		TAGTAGGGACAACAACA
11-Sense	8047–8952	GTTGAGGCACTTTGTGA
11 -Antisense		AGACACCTTCAACACCC
12-Sense	8409–9353	GAGAAACACGGAAACTA
12-Antisense		TACTGAAGATCACACCC

Non-isotopic RNase cleavage assay (NIRCA)

The MisMatch Detect II Non-Isotopic RNase Cleavage Assay Kit (Ambion, Austin, TX) was used to screen the samples for *ATM* mutations [28, 29]. Each second stage PCR product was transcribed in two reaction mixes using 4 μl of PCR product, 250 nM rNTPs, 1 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris-Cl, pH 7.5, 7 mM $MgCl_2$,

2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 12 µl with RNase free H_2O. Following incubation for 90 min at 37°C, 6 µl of buffer containing 80% formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0, was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95°C for 5 min and allowed to cool at room temperature for 5 min. The duplex reactions of 4.5 µl were mixed with 13.5 µl of RNase digestion buffer (10 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with RNase 2 and RNase 1 (from *E. coli*) at a concentration of 1:100 (0.5 µg/ml) and 1:200, respectively. The reaction mixtures were incubated at 37°C for 25 min and resolved on a 2% agarose gel run at 100 V for approximately 45 min.

Table 3. Sequences of second stage ATM primers

Fragment number	Bases amplified	Primer sequence[a]
1-T7	37–790	TAATCGACTCACTATAGGG
		CGGTTGATACTACTTTG
1-SP6		ATTTAGGTGACACTATAGAA
		TTTAATCCGTCAGTCT
2-T7	755–1632	TAATCGACTCACTATAGGA
		ATAATTCATGCTGTTAC
2-SP6		ATTTAGGTGACACTATAGAA
		TTTTATTCCAGAGTTT
3-T7	1570–2425	TAATCGACTCACTATAGGA
		AGTTGCATTGTGTCAAG
3-SP6		ATTTAGGTGACACTATAGGA
		GTTGGCTTTCTGGAA
4-T7	2368–3247	TAATCGACTCACTATAGGG
		CTGCTACTGTTACA
4-SP6		ATTTAGGTGACACTATAGAA
		TGCTCCAATTACTGT
5-T7	3055–3944	TAATCGACTCACTATAGGA
		GAAGAGTACCCCTTGC
5-SP6		ATTTAGGTGACACTATAGGA
		GATGTGGAATCAAAACCTTAT
6-T7	3911–4796	TAATCGACTCACTATAGGA
		CACAAATATTGAGGAT
6-S P6		ATTTAGGTGACACTATAGAA
		GTCCAATACCTGTT
7-T7	4782–5644	TAATCGACTCACTATAGGG
		TGGAGGTTCAGA
7-SP6		ATTTAGGTGACACTATAGAA
		GAATTTCACATTTTGT
8-T7	5607–6500	TAATCGACTCACTATAGGA
		AAGACACTGACTTGTG
8-SP6		ATTTAGGTGACACTATAGAA
		CTGCATATTCCTCC
9-T7	6451–7285	TAATCGACTCACTATAGGA
		ATAAAGACTGGTGTCC
9-S P6		ATTTAGGTGACACTATAGAA
		TTTCCAGCAACTTC
10-T7	7213–8113	TAATCGACTCACTATAGGG
		CAACTGGTTAGC
10-SP6		ATTTAGGTGACACTATAGAA
		TATTTATGCCTTTTCT
11-T7	8082–8669	TAATCGACTCACTATAGGA
		AACTTAGATGCCACTC
11-SP6		ATTTAGGTGACACTATAGGA
		AAACTGGTTGAA
12-T7	8598–9328	TAATCGACTCACTATAGGA
		AAATGATGGAGGTGC
12-SP6		ATTTAGGTGACACTATAGAA
		TCCTGGGAAAAGTCG

[a]The underlined regions indicate the phage promoter sequences. Generally, the first two bases at the 3′ end of the promoter overlapped in each case with the first two bases of the *ATM* fragment.

Results

RNA was isolated from peripheral blood lymphocytes of 37 breast cancer patients in order to detect possible *ATM* mutations. The characteristics of the subjects are presented in Table 1. cDNA copies of the extracted mRNA were produced for each sample. This approach was employed, rather than the use of genomic DNA, to avoid analysis of intronic DNA regions. The *ATM* open reading frame was amplified in 12 overlapping segments using nested PCR. The products of the second stage PCR reactions, all roughly 1 kb in size, were subjected to agarose gel electrophoresis and the results obtained for three patients using two sets of primers and a *p*53 control displayed in Figure 1.

The PCR products that contained promoters for either the T7 or SP6 RNA polymerase were then transcribed in an *in vitro* system. The convention followed with this assay was linkage of the *T7* promoter to the sense strand of the fragment while the *SP6* promoter was attached to the antisense strand. The RNA product produced from either the sense or antisense strand was hybridized with the complementary RNA strand produced using known normal tissue not possessing an *ATM* mutation. In addition, for each experiment, the

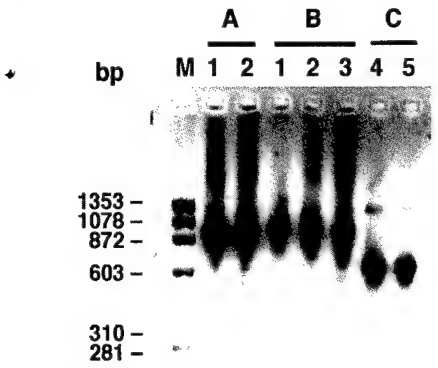


Figure 1. PCR products. Second stage PCR products for DNA isolated from two breast cancer patients (lanes 1 and 2) and one person not diagnosed with breast cancer (lane 3) and amplified with primers for fragments 4 and 6, labeled as A and B, respectively. DNA known not to possess a mutation in the *p*53 gene and a DNA sample with a known mutation are shown in lanes 4 and 5, respectively. These samples were amplified with primers for a DNA fragment encompassing *p*53 exons 5 and 6 and labeled C.

RNA produced from a known mutant and wild type was hybridized with RNA transcribed from a wild type DNA template to serve as positive and negative controls for this assay, respectively.

These duplexes were subjected to agarose gel electrophoresis either prior to (Figure 2) or following digestion (Figure 3) with RNase. The basis of this screening technique is the sensitivity to cleavage with RNase of an RNA duplex containing a mismatched region [28, 29]. This heteroduplex forms when RNA produced from a PCR product possessing a mutation at a particular site is hybridized with RNA produced from a wild type DNA template. As can be seen in Figure 3, there was the appearance of two bands following

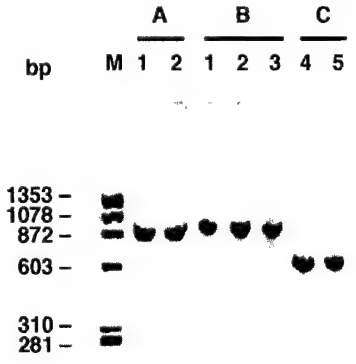


Figure 2. Transcription products – no digestion. RNA products resulting from hybridization of RNA produced from the sense strand of tumor biopsy DNA and RNA synthesized from the antisense strand of DNA isolated from normal human DNA. The lanes are labeled as described in Figure 1.

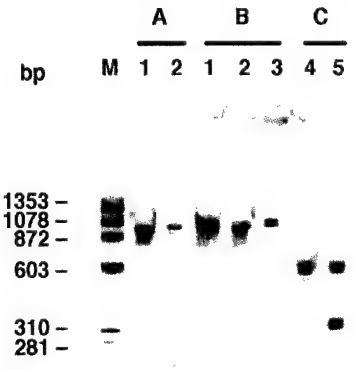


Figure 3. RNase-treated transcription products. Products resulting from RNase digestion of the duplex RNAs shown in Figure 2.

digestion of the RNA duplex derived from DNA fragments containing a known mutation in the *p*53 gene confirming the presence of a mutation. In contrast, none of the assays performed to detect *ATM* mutations exhibited multiple bands, other than for samples obtained from eight patients in which the common G→A transition polymorphism at base 5557 in exon 39 (30–32) was detected. These results indicate that there were no *ATM* mutations present in the breast cancer patients tested.

Discussion

A series of 37 breast cancer patients was screened for germline mutations in the *ATM* gene. This work was accomplished using NIRCA, an assay that is complementary to the approaches which have been previously used to estimate *ATM* mutation frequency in breast cancer patients. No *ATM* mutations were detected in this study, which is consistent with the results obtained using other techniques [22–24]. Although the number of patients tested in this study was modest, our finding of no *ATM* mutations in a group of 37 patients provides a 95% upper confidence interval limit for *ATM* carriers among breast cancer patients of 7.8%.

NIRCA was used in this study for a variety of reasons:

(1) It has been shown that NIRCA is capable of detecting a variety of mutations, including point mutations as well as deletions and insertions, with a high level of sensitivity and accuracy [28, 29, 33–40];

(2) Relatively large target regions of at least 1000 bp can be screened in a single step. This is in contrast to other assays that are limited to much smaller target regions. This is critical for screening *ATM*, as

even the expressed portion of the gene is large, and the identified mutations are scattered throughout the gene;

(3) Using NIRCA, it is not necessary to possess any information as to potential sites for mutations. The entire coding sequence of the gene can be analyzed and therefore mutations at any point along the gene can be detected;

(4) The position of the bands on a gel provide a preliminary identification of the location for a mutation which facilitates DNA sequencing and analysis of sequencing data.

Acknowledgements

This research was supported by grant DAMD17-97-1-7208 from the Breast Cancer Research Fund of the Department of Defense and the Mount Sinai Breast Cancer Advisory Board. Barbara Ksienski is thanked for her technical assistance.

References

1. Shiloh Y: Ataxia telangiectasia: closer to unraveling the mystery. Eur J Hum Genet 3:116–138, 1995
2. Morell D, Cromartie E, Swift M: Mortality and cancer incidence in 263 patients with ataxia-telangiectasia. J Natl Cancer Inst 77: 89–92, 1986
3. Gatti RA, Berkel I, Boder E, Braedt C, Charmley P, Concannon P, Ersoy F, Foroud T, Jasper NGJ, Lange K, Lahtrop GM, Leppert M, Nakamura Y, O'Connell P, Paterson M, Salser W, Sanal O, Silver J, Sparkes RS, Susi E, Weeks DE, Wei S, White R, Yoder F: Localization of an *ataxia-telangiectasia* gene to chromosome 11q22-23. Nature 336: 577–580, 1988
4. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Seez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor AMR, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS, Shiloh Y: A single gene with a product similar to PI3-kinase and Rad 3 is mutated in ataxia telangiectasia. Science 268: 1749–1753, 1995
5. Telatar M, Teraoka S, Wang Z, Chun HH, Liang T, Castellvi-Bell S, Udar N, Borresen-Dale AL, Chessa L, Bernatowska-Matuzkievicz E, Porras O, Watanabe M, Junker A, Concannon P, Gatti R: Ataxia-telangiectasia: identification and detection of founder-effect mutations in the *ATM* gene in ethnic populations. Am J Hum Genet 62: 86–97,1998
6. Concannon P, Gatti A: Diversity of *ATM* gene mutations detected in patients with ataxia-telangiectasia. Hum Mutat 10: 100–107, 1997
7. Gotoff SP, Amirmokri E, Liebner EJ: Ataxia-telangiectasia. Am J Dis Child 114: 617–625, 1967
8. Dhand R, Hues I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, Kasuga M, Courtneidge SA, Waterfield MD: PI3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO J 13: 522–533, 1994
9. Tanti JF, Gremeaux T, Wan Obberghen F, LeMarchand-Brustel Y: Insulin receptor substrate 1 is phosphorylated by the serine kinase activity of phosphatidylinositol 3-kinase. Biochem J 304: 17–21, 1994
10. Freund GG, Wittig JG, Mooney RA: The PI3-kinase serine kinase phosphorylates its p85 subunit and IRS-1 in PI3-kinase/IRS1-complexes. Biochem Biophys Res Commun 206: 272–278, 1995
11. Harnden DG: The nature of ataxia-telangiectasia: problems and perspectives. Int J Radiat Biol 66: S13–S19, 1994
12. Taylor AMR, Byrd PJ, McConville CM, Thacker S: Genetic and cellular features of ataxia-telangiectasia. Int J Radiat Biol 65: 65–70, 1994
13. Swift M, Morell D, Cromartie E, Chamberlin AR, Skolnik MH, Bishop DT: The incidence and gene frequency of ataxia-telangiectasia in the United States. Am J Hum Genet 39: 573–583, 1986
14. Chen PC, Lavin MF, Kidson C, Moss D: Identification of ataxia-telangiectasia heterozygotes, a cancer prone population. Nature 274: 484–486, 1978
15. Weeks DE, Paterson MC, Lange K, Andrais B, Davis RC, Yoder F, Gatti RA: Assessment of chronic γ radiosensitivity as an *in vitro* assay for heterozygote identification of ataxia-telangiectasia. Radiat Res 128: 90–99, 1991
16. Borresen AL, Anderson TI, Treti S, Heiberg A, Moller P: Breast cancer and other cancers in Norwegian families with ataxia-telangiectasia. Genes Chrom Cancer 2: 339–340, 1990
17. Swift M, Morrell D, Massey RB, Chase CL: Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med 325: 1831–1836, 1991
18. Athma P, Rappaport R, Swift M: Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer. Cancer Genet Cytogenet 92: 130–134, 1996
19. Swift M, Reitnauer PJ, Morel D, Chase CL: Breast and other cancers in families with ataxia-telangiectasia. N Engl J Med 316: 1289–1294, 1987
20. Easton DF: Cancer risk in ataxia-telangiectasia heterozygotes. Int J Radiat Biol 66: S177–S182, 1994
21. Pippard EC, Hall AJ, Barker DJP, Bridges BA: Cancer in homozygotes and heterozygotes of ataxia-telangiectasia and xeroderma pigmentosum in Britain. Cancer Res 48: 2929–2932, 1988
22. Vorechovsky I, Rasio D, Luo L, Monaco C, Hammarstrom L, Webster ADB, Zaloudik J, Barbanti-Brodano G, James M, Russo G, Croce CM, Negrini M: The *ATM* gene and susceptibility to breast cancer: analysis of 38 breast tumors reveals no evidence for mutation. Cancer Res 56: 2726–2732, 1996
23. Bay JO, Grancho M, Pernin D, Presneau N, Rio P, Tchirkov A, Uhrhammer N, Verrelle P, Gatti RA, Bignon YJ: No evidence for constitutional *ATM* mutation in breast/gastric cancer families. Int J Oncol 12: 1385–1390, 1998
24. Chen J, Giesler-Birkholtz G, Lindblom P, Rublo C and Lindblom A: The role of ataxia-telangiectasia heterozygotes in familial breast cancer. Cancer Res 58: 1376–1379, 1998
25. Appleby JM, Barber JB, Levine E, Varley JM, Taylor AM, Stankovic T, Heighway J, Warren C, Scott D: Absence of mutations in the *ATM* gene in breast cancer patients with severe responses to radiotherapy. Br J Cancer 76: 1546–1549, 1997

26. Clarke RA, Goozee GR, Birrell G, Fang ZM, Hasnain H, Lavin M, Kearsley JH: Absence of *ATM* truncations in patients with severe acute radiation reactions. Int J Radiation Oncol Biol Phys 41: 1021–1027, 1998
27. Ramsay J, Birrell G, Lavin M: Testing for mutations of the *ataxia-telangiectasia* gene in radiosensitive breast cancer patients. Radiother Oncol 47: 125–128, 1998
28. Goldrick MM, Kimball GR, Liu Q, Martin LA, Sommer SS, Tseng JY-H: NIRCA: A rapid robust method for screening for unknown point mutations. BioTechniques 21: 106–112, 1996
29. Watkins HC, M Goldrick: Cleavage of RNA–RNA hybrids at mutation sites using the nonisotopic RNase cleavage assay (NIRCA). In: Dracopoli NC et al. (eds) Current Protocols in Human Genetics. Wiley, New York 1998, pp 7.2.5–7.2.18.
30. Castellvi-Bel S, Sheikhavandi S, Telatar M, Tai L-Q, Hwang M, Wang Z, Yang Z, Cheng R, Gatti RA: New mutations, polymorphisms, and rare variants in the *ATM* gene detected by a novel SSCP strategy. Hum Mutat 14: 156–162, 1999
31. Maillet P, Vaudan G, Chappuis P, Sappino A-P: PCR-mediated detection of a polymorphism in the *ATM* gene. Mol Cell Probes 13: 67–69, 1999
32. Sandoval N, Platzer M, Rosenthal A, Dork T, Bendix R, Skawran B, Stuhrmann M, Wegner R-D, Sperling K, Banin S, Shiloh Y, Baumer A, Bernthlaler U, Sennefelder H, Brohm M, Weber BHF, Schindler D: Characterization of *ATM* gene mutations in 66 *ataxia-telangiectasia families*. Hum Mol Genet 8: 69–79, 1999
33. De Souza AT, Hankins GR, Washington MK, Orton TC, Jirtle RL: *M6P/IGF2R* gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. Nature Genet 11: 447–449, 1995
34. Maslen C, Babcock D, Raghunath M, Steinmann B: A rare branch-point mutation is associated with missplicing of fibrillin-Z in a large family with congenital contractural arachnodactyly. Am J Hum Genet 60: 1389–1398, 1997
35. Tsai T, Davalath S, Rankin C, Radich JP, Head D, Appelbaum FR, Boldt DH: Tumor suppressor gene alteration in adult acute lymphoblastic leukemia (ALL). Analysis of retinoblastoma (*Rb*) and *p*53 gene expression in lymphoblasts of patients with de novo, relapsed, or refractory ALL treated in Southwest Oncology Group studies. Leukemia 10: 1901–1910, 1996
36. Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR: Somatic mutations in the *p*53 tumor suppressor gene in rheumatoid arthritis synovium. Proc Natl Acad Sci USA 94: 10895–10900, 1997
37. Peral B, Gamble V, Strong C, Ong ACM, Sloane-Stanley J, Zerres K, Winearls CG, Harris PC: Identification of mutations in the duplicated region of the polycystic kidney disease 1 gene (*PKD1*) by a novel approach. Am J Hum Genet 60: 1399–1410, 1997
38. Ritis K, Steletas M, Tsiroxidoe V, Pardali E, Kanariol M, Moschese V, Orlandi P,Skordala M, Rossi P, Kartalis G, Bourikas G, Sideras P: Absence of Bruton's tyrosine kinase (*Btk*) mutations in patients with acute myeloid leukaemia. Br J Haematol 102: 1241–1248, 1998
39. Toyoshima M, Hara T, Zhang H, Yamamoto T, Akaboshi S, Nanba E, Ohno K, Hori N, Sato K, Takeshita K: Ataxia-telangiectasia without immunodeficiency: novel point mutations within and adjacent to the phosphatidylinositol 3-kinase-like domain. Am J Med Genet 75: 141–144, 1998
40. Macera MJ, Godec CJ, Sharma N, Verma RS: Loss of heterozygosity of the *TP53* tumor suppressor gene and detection of point mutations by the non-isotopic RNAse cleavage assay in prostate cancer. Cancer Genet Cytogenet 108: 42–47, 1999

Address for offprints and correspondence: Dr. Barry S. Rosenstein, Box 1236, Department of Radiation Oncology, Mount Sinai School of Medicine, New York, NY 10029; *Tel.:* (212)241-9408; *Fax:* (212)996-8927

SUBMITTED FOR PUBLICATION

American Journal of Human Genetics

Report

Identification of an Individual Possessing Two Mutated Copies of the *ATM* Gene but not Exhibiting Symptoms of the Disorder Ataxia Telangiectasia

David P. Atencio,[1] Christopher M. Iannuzzi,[1] Kevin M. Hopkins[2], Jonine L. Bernstein[3], Howard B. Lieberman[2] and Barry S. Rosenstein[1, 3, 4,5]

[1]Departments of Radiation Oncology, [3]Community & Preventive Medicine and [4]Dermatology, Mount Sinai School of Medicine, New York, NY; [2]Center for Radiological Research, Columbia University, New York, NY; [5]Department of Radiation Oncology, New York University School of Medicine, New York, NY

It has long been held that a person harboring mutations in both copies of the *ATM* gene will display the disorder ataxia telangiectasia (AT). However, we have identified a woman who does not manifest any of the classic symptoms of this disease but possesses non-conservative missense mutations in both copies of the *ATM* gene. One mutation is located in exon 30 (4138C→T) and causes replacement of a tyrosine for a histidine. The second mutation was discovered in exon 31 (4400A→G) and encodes an amino acid change from aspartate to glycine. Both amino acid substitutions are of sufficient structural difference so as to predict for a potential alteration in ATM protein function. Four intronic polymorphisms were also identified. Although this individual lacks the typical AT phenotype, she was diagnosed with breast cancer and experienced enhanced radiation-related late morbidity following radiotherapy manifested by increased skin hyperpigmentation and subcutaneous fibrosis. To the best of our knowledge, this is the first person identified who possesses two mutated copies of *ATM*, but does not exhibit classical AT-associated characteristics. This demonstrates that the presence of missense mutations in both copies of *ATM* is not sufficient for the phenotypic manifestation of AT, although it may result in cancer susceptibility and enhanced adverse radiotherapy reactions.

Ataxia telangiectasia (AT) is a rare autosomal recessive disorder that appears thus far only when both copies of the *ATM* gene are mutated. This disorder has a prevalence of approximately 1/100,000 (Swift et al. 1996; Woods et al. 1990) and is characterized by a pleiotropic phenotype involving multiple organ systems (Segdwick and Boder 1991). Progressive cerebellar degeneration, immunodeficiency, chromosomal instability, cancer predisposition, gonadal dysgenesis and oculocutaneous telangiectasias are typical manifestations of this genetic disorder. The gene responsible, *ATM*, was cloned and its DNA sequence determined in 1995 by Savitsky and coworkers. It is located on chromosome 11q22-23 (Gatti et al. 1988; Lange et al. 1996) and contains 66 exons with start and stop codons located within exons 4 and 65, respectively. The product of the *ATM* gene is a ubiquitous 370kDa (Chen and Lee 1996) nuclear phosphoprotein (Watters et al. 1997) that functions primarily as a protein kinase (Brunn et al. 1996; Banin et al. 1998; Canman et al. 1998; Sarkaria et al. 1998). The ATM protein participates in the regulation of cell cycle progression and the maintenance of genomic integrity. Cells from AT patients are radiosensitive (Cole et al. 1988) and deficient in several cell cycle checkpoints induced by ionizing radiation exposure (Kastan et al. 1992; Takagi et al. 1998).

In contrast to the rarity of the AT disorder, it has been suggested that 1-2% (Swift et al. 1986) of the overall population are heterozygous for a single *ATM* mutation and that people who are *ATM* mutation carriers are more susceptible to the development of several forms of cancer (Vorchevsky et al. 1997; Stankovic et al. 1999; Schaffner et al. 2000). Furthermore, it has been suggested that cancer susceptibility is associated with the presence of a missense mutation (Gatti et al. 1999) and resultant decreased levels of ATM protein or diminished protein activity may cause inadequate genomic surveillance. AT patients, however, have truncation mutations in the

majority of cases (Concannon and Gatti 1997) and the ATM protein is often absent (Sandoval et al. 1999). A hallmark of AT patients is their marked sensitivity to ionizing radiation (Gotoff et al. 1967; Taylor et al. 1975) and this has led to speculation that *ATM* heterozygotes are prone to radiation-related complications resulting from radiotherapy (Varghese et al. 1999).

A peripheral blood sample was obtained from the person who is the subject of this study, and lymphocytes were isolated by Ficoll-Paque separation. DNA was extracted and PCRs were performed to amplify each of the 62 *ATM* exons that comprise the translated region of the gene. The PCR products were screened for *ATM* base sequence alterations using a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) to perform denaturing high performance liquid chromatography (DHPLC). The buffer gradient and temperature conditions were calculated using WAVEmaker software (Transgenomic, v3.3). For those products that exhibited a variant chromatogram, the PCR was repeated and the sequence of both DNA strands was determined using an ABI 3700 DNA sequencer (Foster City, CA).

The patient under study is a 77-year-old post-menopausal African-American female first diagnosed with breast cancer in 1989. There was no family history of breast cancer. She received a right breast lumpectomy and axillary node dissection that revealed a 3 cm moderately differentiated invasive ductal carcinoma. Sixteen axillary lymph nodes were removed and proved negative for metastatic carcinoma. The tumor was estrogen receptor negative and progesterone receptor positive. She underwent a course of adjuvant chemotherapy consisting of six cycles of CMF (cyclophosphamide/methotrexate/5-FU), followed by tamoxifen for 5 years. The patient was followed with annual mammography and did well without evidence of disease until an

abnormal mammogram was found in 1997. An excision revealed intermediate grade DCIS and a small (0.2 cm) area of microinvasive ductal carcinoma. The patient received a course of adjuvant radiation therapy consisting of 45 Gy to the whole breast, followed by an electron boost of 14 Gy to the tumor bed. She tolerated radiation therapy without incident, experiencing only a routine RTOG acute grade 1 reaction (Cox et al. 1995). However, a significant RTOG late grade 2 skin and subcutaneous tissue complication characterized by fibrosis of the right breast as well as persistent hyperpigmentation and edema became evident two years following radiation therapy. This late reaction was more severe than one would expect based on the modest skin reaction noted during treatment.

DHPLC screening of this individual revealed 6 aberrant chromatograms. The sequence of the DNA from the variant exons was determined and revealed four intronic polymorphisms, as well as two non-conservative missense mutations. The DHPLC and sequencing chromatograms for both wild type DNA and PCR products for regions containing the two missense mutations are shown in figure 1A. The polymorphisms detected were IVS17-56G→A, IVS19-17G→T, IVS40+26C→T and IVS48-69insATT. The missense mutations identified in exons 30 and 31, 4138C→T and 4400A→G, were non-conservative and caused the substitution of tyrosine for a histidine and glycine in place of an aspartate, respectively.

Further analysis was conducted to ascertain the cis/trans arrangement of the two non-conservative missense mutations. It was possible to produce a PCR product encompassing both of these mutations as they are located in adjacent exons and the intron dividing these exons is relatively small (498 bp). The forward primer for exon 30 and the reverse primer for exon 31

were employed to amplify a 953-bp product that included both exons. The fragment was cloned into a pGEM-T vector (Promega), and transformed into *E. coli* DH5α. The transformed cells were spread on LB plates containing 100μg/ml ampicillin (LB+amp) and spread with X-gal, then incubated overnight at 37°C. White colonies were picked and re-spread to obtain single colonies and avoid the possibility of mixed colonies resulting from cloning a heteroduplex PCR product. Single colonies were picked and transferred to liquid YT+amp (100μg/ml) media and incubated overnight, shaking at 37°C. Plasmid DNA was obtained from the clones using a miniprep kit (Qiagen). A diagnostic *Bam*HI digestion was performed to confirm the presence and ascertain the orientation of the cloned insert.

The sequence of the DNA from six plasmids was determined. Sequencing chromatograms for the region surrounding each mutation are displayed in figure 1B for two plasmids and wild type DNA. The sites of the mutated alleles are marked in the wild-type sequence with an arrow. All of the plasmid sequences fell into two categories represented as mut-1 and mut-2. All of the mut-1 plasmids displayed the C→T transition in exon 30 and a wild type exon 31 sequence whereas all of the mut-2 plasmids exhibited the A→G transition in exon 31 and a wild type exon 30 sequence. None of the plasmids possessed either both mutations or exhibited no mutation. This demonstrates that each mutation was located on a different chromosome.

The four polymorphisms likely do not mediate a change in either ATM protein structure or function given their locations within the intron, outside of predicted splice site regions. The two exonic mutations, however, with their respective nonconservative amino acid substitutions probably change ATM protein structure as the amino acid side chain alterations are significant

and could result in conformational alterations that compromise function. However, neither of these two mutations is located within regions of the gene that encode critical functional domains of the ATM protein. Hence, these amino acid substitutions may have only a modest impact on ATM protein function. It has been demonstrated using ATM antisera that some AT patients possessing a missense mutation continue to synthesize a functional protein, although at low levels (Lakin et al. 1996). Consistent with this limited affect upon protein activity, a reduced rate of disease progression was noted in these cases. Moreover, McConville et al. (1996) identified 14 AT families and documented milder phenotypes for those AT patients having a missense mutation at one allele. If the patient described in this report has the capacity to produce even a moderately functional protein, this may have spared her from the development of classical AT symptoms and only resulted in increased breast cancer susceptibility and enhanced adverse responses to radiotherapy.

Another explanation for preservation of ATM protein function despite the presence of two mutated copies of the gene is intragenic complementation (Crick and Orgel 1964). The individual altered proteins alone may not be independently functional, but if each contains a point mutation in a unique functional domain or in different regions of a single domain, then the two polypeptides together may interact and form a near-normal functional multimeric protein. A phenotype less severe than what would have been predicted had either of the point mutations been homozygous can thus be observed. Moreover, structural studies of the ATM protein using atomic force microscopy (Smith et al. 1999) suggest that the ATM protein exists in more than one conformational state, in particular, a tetrameric conformation thus lending itself to the concept of intragenic complementation.

In conclusion, we report the discovery of a person possessing two mutated copies of the *ATM* gene but not demonstrating classical AT symptoms. The clinical characteristics of this individual are consistent with the hypothesis that truncating mutations in *ATM* result in AT, whereas missense mutations are associated with increased cancer susceptibility and complications from radiotherapy. Functional assays of a lymphoblastoid cell line derived from this person are planned to further elucidate the impact of these mutations on the activity of the ATM protein produced by this individual.

Acknowledgments

This work was supported by Grant Number: DAMD17-97-1-7208 from the Breast Cancer Research Fund of the Department of Defense (BSR) and NIH grant GM52493 (HBL). In addition, support from the Mount Sinai Ruttenberg Cancer Center is gratefully acknowledged which enabled purchase of the WAVE Nucleic Acid Fragment Analysis System.

Refererences

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281:1674-1677

Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC, Abraham RT (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J 15: 5256-5267

Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281:1677-1679

Chen G, Lee EYHP (1996) The product of the ATM gene is a 370kDa nuclear phosphoprotein. J Biol Chem 271:33693-22697

Cole J, Arlett CF, Green MH, Harcourt SA, Priestly, A, Henderson L, Cole H, James SE, Richmond F (1988) Comparative human cellular radiosensitivity: II. The survival following gamma-irradiation of unstimulated (Go) T-lymphocytes, T-lymphocyte lines, lymphoblastoid cell lines and fibroblasts from normal donors, from ataxia-telangiectasia patients, and from ataxia-telangiectasia heterozygotes. Int J Radiat Biol 54:929-943

Concannon P, Gatti RA (1997) Diversity of ATM gene mutations detected in patients with ataxia-telangiectasia. Hum Mutat 10:100-107

Cox JD, Stetz J, Pajak TF (1995) Toxicity criteria of the radiation therapy oncology group (RTOG) and the european organization for research and treatment of cancer (EORTC). IJROBP 31(5):1341-1346

Crick FHC, Orgel LE (1964) The theory of inter-allelic complementation. J Mol Biol 8:161-165.

Gatti RA, Berkel I, Boder E, Braedt G, Charmley P, Concannon P, Ersoy F, Foroud T, Jaspers NG, Lange K (1988) Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. Nature 336:577-580

Gatti RA, Tward A, Concannon P (1999) Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations. Mol Genet Metab 68:419-423

Gotoff SP, Amirmokri E, Liebner EJ (1967) Ataxia-telangiectasia. Neoplasia, untoward response to X-irradiation, and tuberous sclerosis. Am J Dis Childhood 114:617-625

Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587-597

Lakin ND, Weber P, Stankovic T, Rottinghaus ST, Taylor AMR, Jackson SP (1996) Analysis of the ATM protein in wild-type and ataxia-telangiectasia cells. Oncogene 13:2707-2716

Lange E, Borresen AL, Chen X, Chessa L, Chiplunkar S, Concannon P, Dandekar S, Gerken S, Lange K, Liang T (1995) Localization of an ataxia-telangiectasia gene to an

approximately 500- kb interval on chromosome 11q23.1: linkage analysis of 176 families by an international consortium. Am J Hum Genet 57: 112-119

McConville CM, Stonkovic T, Byrd PJ, McGuire GM, Yao QY, Lennox GG, Taylor MR. (1996) Mutations associated with variant phenotypes in ataxia-telangiectasia. Am J Hum Genet 59:320-330

Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT (1998) Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res 58:4375-4382

Sandoval N, Platzer M, Rosenthal A, Dork T, Bendix, R, Skawran B, Stuhrmann M, Wegner RD, Sperling K, Banin S, Shiloh Y, Baumer A, Bernthaler U, Sennefelder H, Brohm M, Weber BHF, Schindler D (1999) Characterization of ATM gene mutations in 66 ataxia telangiectasia families. Hum Mol Genet 8(1):69-79]

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268:1749-1753

Sedgwick RP, Boder E (1991) Ataxia-telangiectasia. In: Vianney De Jong JMB, editor. Hereditary and Spinocerebellar Atrophies. Amsterdam: Elsevier, Inc. pp 347-423

Shafman T, Khanna KK, Kedar P, Spring K, Kozlov S, Yen T, Hobson K, Gatei M, Zhang N, Watters D, Egerton M, Shiloh Y, Kharbanda S, Kufe D, Lavin MF. (1997) Interaction between ATM protein and c-Abl in response to DNA damage. Nature 387:520-523

Smith GC, Cary RB, Lakin ND, Hann BC, Teo SH, Chen DJ, Jackson SP (1999) Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM. Proc Natl Acad Sci USA 96(20):11134-11139

Swift M, Morrell D, Cromartie E, Chamberlin AR, Skolnick MH, Bishop DT (1986) The incidence and gene frequency of ataxia-telangiectasia in the United States. Am J Hum Genet 39:573-583

Takagi M, Delia D, Chessa L, Iwata S, Shigeta T, Kanke Y, Goi K, Asada M, Eguchi M, Kodama C, Mitzutani S (1998) Defective control of apoptosis, radiosensitivity, and spindle checkpoint in ataxia-telangiectasia. Cancer Res 58:4923-4929

Taylor AMR, Harndon DG, Arlett CF, Harcourt SA, Lehman AR, Stevens S, Bridges BA (1975) Ataxia-telangiectasia, a human mutation with abnormal radiation sensitivity. Nature 258:427-429

Varghese S, Schmidt-Ullrich RK, Dritschilo A, Jung M (1999) Enhanced radiation late effects and cellular radiation sensitivity in an ATM heterozygous breast cancer patient. Radiat Onc Invest 7:231-237

Vorechovsky I, Luo L, Dyer MJ, Catovsky D, Amlot PL, Yaxley JC, Foroni L, Hammarstrom L, Webster AD, Yuille MA (1997) Clustering of missense mutations in the ataxia-telangiectasia gene in a sporadic T-cell leukaemia. Nat Genet 17:96-99

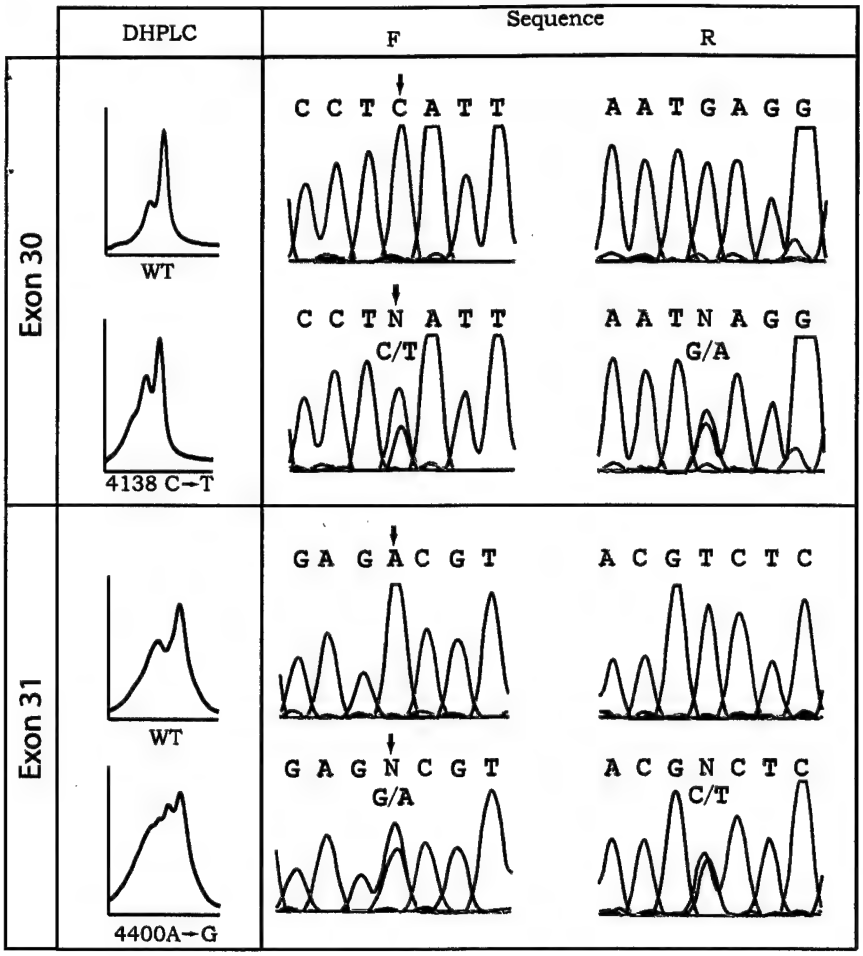
Watters D, Khanna KK, Beamish H, Birrell G, Spring K, Kedar P, Gatei M, Stenzel D, Hobson K, Kozlov S, Zhang N, Farrell A, Ramsay J, Gatti R, Lavin M (1997) Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. Oncogene 14:1911-1921

Woods CG, Bundey SE, Taylor AMR (1990) Unusual features in the inheritance of ataxia-telangiectasia. Hum Genetics 84:555-562

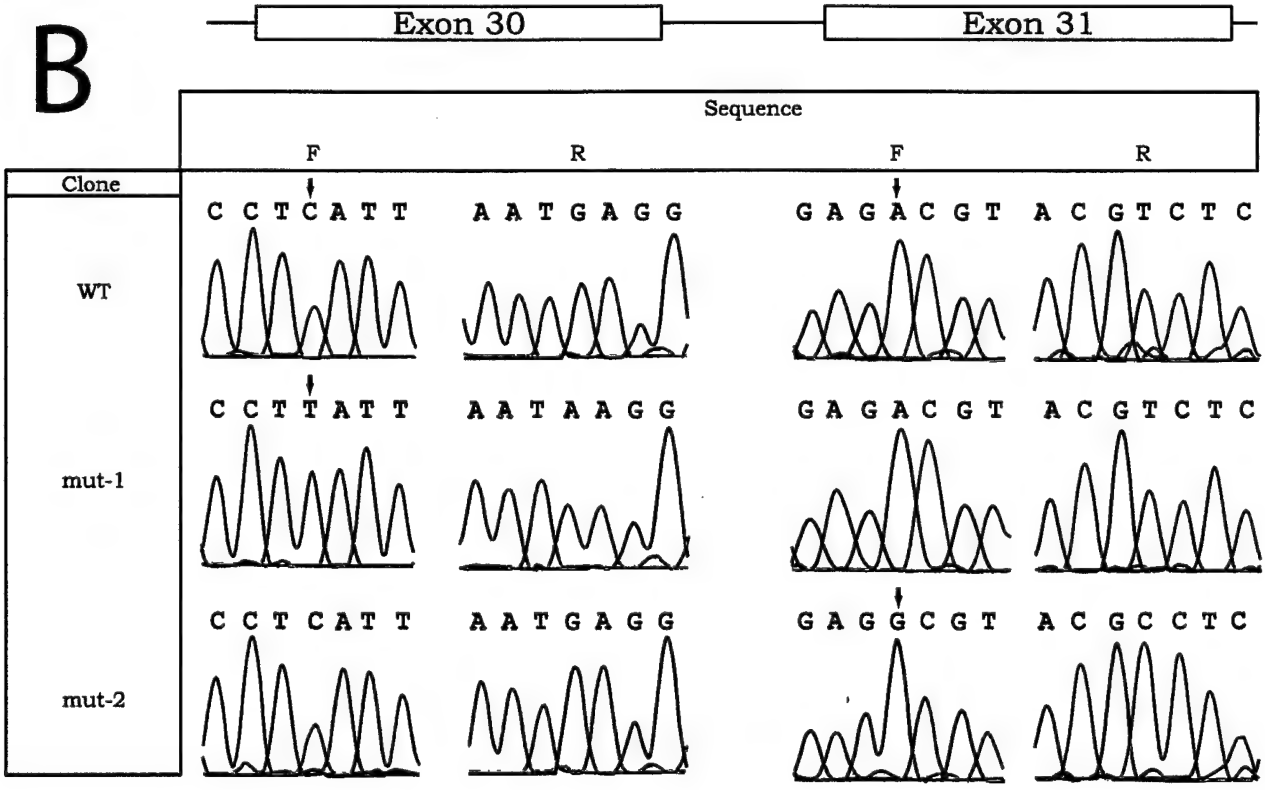
Figure Legend

Figure. 1. (A) The DHPLC chromatogram and sequencing profiles for the exon 30 and exon 31 PCR products. F designates the forward sequence and R is the confirmatory reverse sequence (reverse compliment of the forward sequence). The arrows indicate the nucleotide in the forward sequence that is mutated in each exon. The double nucleotide band presented in the mutated DNA appears as a double peak and the nucleotides present on each chromosomal copy of the gene are indicated below the N in each case. (B) Sequencing chromatograms for the region surrounding the C→T transition in exon 30 and the A→G transition in exon 31of the mut-1 and mut-2 plasmids, respectively, as well as WT (wild type) DNA.

A


DHPLC
Sequence
F
R
Exon 30
WT
4138 C→T
C C T C A T T
A A T G A G G
C C T N A T T
C/T
A A T N A G G
G/A
Exon 31
WT
4400A→G
G A G A C G T
A C G T C T C
G A G N C G T
G/A
A C G N C T C
C/T

B


Exon 30
Exon 31
Sequence
F
R
F
R
Clone
WT
mut-1
mut-2
C C T C A T T
A A T G A G G
G A G A C G T
A C G T C T C
C C T T A T T
A A T A A G G
G A G A C G T
A C G T C T C
C C T C A T T
A A T G A G G
G A G G C G T
A C G C C T C

SUBMITTED FOR PUBLICATION

International Journal of Radiation Oncology Biology Physics

ATM MUTATIONS IN FEMALE BREAST CANCER PATIENTS PREDICT FOR AN INCREASE IN RADIATION-INDUCED LATE EFFECTS

Christopher M. Iannuzzi MD; David P. Atencio PhD, Sheryl Green MD, Richard G. Stock MD, and Barry S. Rosenstein PhD
Department of Radiation Oncology, Mount Sinai School of Medicine, New York, NY

Grant sponsor: Breast Cancer Research Fund of the Department of Defense; Grant Number: DAMD17-97-1-7208.

*Correspondence to: Christopher M. Iannuzzi, MD, Department of Radiation Oncology, Box 1236, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: christopher.iannuzzi@mssm.edu

ABSTRACT

Purpose: Mutation of the *ATM* gene may be associated with enhanced radiosensitivity and increased radiation-induced morbidity. Denaturing high performance liquid chromatography (DHPLC) is a powerful new technique proven to be sensitive and accurate in the detection of missense mutations as well as small deletions and insertions. We screened female breast cancer patients for evidence of *ATM* gene alterations using DHPLC. This study attempts to determine whether breast cancer patients who develop severe radiotherapy-induced effects are more likely to possess *ATM* mutations compared with patients who display normal radiation responses.

Methods: 46 patients with early stage breast carcinoma underwent limited surgery and adjuvant radiation therapy. DNA was isolated from blood lymphocytes and each exon of the *ATM* gene was amplified using PCR. Genetic variants were identified using DHPLC by comparing test patterns with a known wild-type pattern. All variants were subjected to DNA sequencing and compared with wild-type sequences for evidence of a mutation. A retrospective review was performed and the RTOG/EORTC acute and late morbidity scoring schemes for skin and subcutaneous normal tissues were applied to quantify radiation-induced effects.

Results: 9 *ATM* mutations were identified in 6 patients (8 novel and 1 rare). The median follow-up was 3.2 years (range: 1.3-10.3 years). A significant correlation between *ATM* mutation status and the devlopment of grade 3-4 subcutaneous

late effects was found. 100% (3/3) of patients that manifested grade 3-4 subcutaneous late sequelae possessed *ATM* mutations whereas only 7% (3/43) of patients who did not develop this form of severe toxicity harbored an *ATM* mutation ($p<0.001$). One *ATM* mutation carrier developed grade 4 soft tissue necrosis after radiation therapy and required hyperbaric oxygen. All 3 patients manifesting grade 3-4 late subcutaneous responses in fact harbored two *ATM* mutations. In contrast, none of the three *ATM* carriers that had a single mutation developed a severe subcutaneous reaction. *ATM* mutation status did not predict for a significant increase in early effects. 17% (4/23) of patients with grade 2-3 moist desquamation had an *ATM* mutation compared with 9% (2/23) of patients without desquamation ($p=0.4$).

Conclusions: Possession, particularly of two *ATM* mutations, is predicitve of an increase in subcutaneous late tissue effects following radiation therapy for breast cancer and may prove to be a relative contraindication to standard management. These patients could be better served with reduced doses of radiation. Equivalent local control remains to be tested but this germline alteration may radiosensitize both tumor and normal tissues. DHPLC is effective in the identification of these patients. A larger study is required to confirm these findings.

Key words: *ATM* gene; Radiation sensitivity; DHPLC; Breast Cancer

INTRODUCTION

Ataxia telangiectasia (AT) is a rare autosomal recessive disorder that results from mutations in both copies of the *ATM* gene. This disorder was first described almost fifty years ago and is characterized by a pleiotropic phenotype and multiple organ system involvement (1). Cerebellar degeneration, ocular telangiectasias, immunodeficiency, and enhanced sensitivity to ionizing radiation are typical manifestations (2). Devastating radiation responses in these patients have also been known for decades (3). A single mutated copy of the *ATM* gene (*ATM* heterozygosity) occurs in approximately 1% of the general population (4). These individuals do not express the classic symptomatology of ataxia-telangiectasia but may be at an increased risk for carcinogenesis and radiosensitivity due to differences in *ATM* protein function or transcription levels (5-7). It has also been postulated that this *ATM* heterozygous state is more common in breast cancer patients compared to that of the general population (8,9).

The *ATM* gene product has been the subject of intensive investigation and appears to act as a central component in cell cycle regulation and genomic stability. It is located primarily in the nucleus of dividing cells (10) and displays protein kinase activity in response to double strand DNA damage (11-13). The downstream effects of *ATM* are critical to the genetic integrity of the cell. This phosphorylation activity has been well characterized at the G1/S checkpoint and

phosphorylation of p53 by *ATM* is a necessary step in cell cycle arrest in response to genomic damage. There is evidence that the ATM protein is also involved at the other cell cycle regulation points (14-16). Cells lacking the ability for proper genomic surveillance and DNA double strand damage repair are at risk for mutagenesis and enhanced radiation responses (17,18).

The hypothesis that cells with an *ATM* mutation exhibit enhanced radiosensitivity has been demonstrated on a cellular level (6,7). Studies have examined cell lines from normal patients, *ATM* heterozygotes, and AT patients and the Do values for *ATM* heterozygotes falls between that of patients with a normal genotype and those with AT (6,19). It is predicted that this inherent radiosensitivity would result in enhanced normal tissue damage after a course of radiation therapy. In vivo studies in female breast cancer patients have failed to correlate *ATM* mutation status to enhanced normal tissue damage (20-24). However, these studies have relied on the protein truncation (PTT) assay that will only detect genetic alterations that result in protein truncation. Mutations that result in early termination of the *ATM* gene product are characteristic of both allelic products in the disorder AT but it has been hypothesized that misense mutations resulting in amino acid substitutions rather than protein truncation are more prevalent in *ATM* heterozygous breast cancer patients (25-27).

Denaturing high performance liquid chromatography (DHPLC) is a robust technique that can be used to screen any gene in a large population for single nucleotide substitutions (28-36). This mutation detection method has proven to

be highly sensitive and specific and will allow for identification of the misense mutations that appear to be most prevalent in *ATM* carriers (9).

This study involves the screening of a group of female breast cancer patients following breast conservation management and attempts to correlate *ATM* germline mutation status with acute and/or late radiation normal tissue effects using DHPLC technology.

METHODS

Patients

Peripheral venous blood samples were collected from 46 female patients with AJCC (37) stage 0-II breast carcinoma who underwent limited surgery and adjuvant radiation therapy between 1992 and 1997. Nine patients also received adjuvant sequential chemotherapy. Patient demographics and tumor characteristics are presented in Table 1. Radiation therapy was delivered with 6 MV photons using either opposed tangential portals alone or 3 fields that included an additional supraclavicular portal if there were positive axillary lymph nodes. Whole breast doses ranged from 4500-5040cGy and the majority (85%) of patients received an electron boost to the surgical bed. Electron energy was typically 12MeV and prescribed to the 85% isodose line. The boost was designed to bring the total dose to above 6000cGy. Wedges were used to increase dose homogeneity. 2D plans were constructed and dose inhomogeneity was kept below 10% for the majority of cases.

All 62 coding exons for the *ATM* gene were screened for a germline mutation using DHPLC. A retrospective chart review was performed and morbidity data was collected. Acute and late skin and subcutaneous tissue reactions were graded according to RTOG/EORTC morbidity criteria (38) (Table 2).

The nature of the project was discussed with each patient and informed consent was obtained prior to obtaining each blood sample as per IRB protocol guidelines.

DNA Isolation

Lymphocytes were isolated by Ficol separation. 1.5 ml Ficol was added to 3 ml of blood and centrifuged at 12000 rpm for 20 min at room temperature. The lymphocytes layer was collected, washed with 10 ml PBS and centrifuged for 10 min, room temperature, at 12,000 rpm.

To isolate DNA from the lymphocytes, 1 ml DNA STAT-60 (Tel-Test, Friendswood, TX) was added to whole blood, followed by addition of 200 µl of chloroform and centrifuged at 4°C for 15 min at 12,000 rpm. The aqueous layer was transferred to a fresh 1.5 ml tube and the extraction procedure repeated. Following an additional chloroform extraction, 500 µl of isopropanol was added to the supernatant. The tube was held at -20°C overnight and centrifuged 10 min at 4°C. The supernatant was removed and the pellet washed with 200 µl of 70% ethanol. After centrifugation at 4°C, 12,000 rpm, for 8 min, the supernatant was removed and the ethanol wash/precipitation was repeated. The supernatant was removed, the pellet air-dried and 150 µl H_2O and 1.5 µl TE buffer was added.

PCR

50 µl PCR reactions were performed to amplify each of the 62 *ATM* exons that comprise the coding region of the *ATM* gene using primers previously described (39). Each PCR reaction contained 100 ng of genomic DNA, 200 µM of each dNTP, 250 nM primers, 2.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA) in Qiagen 10X buffer diluted to 1X. RNase free water was then

added to a total volume of 50 µl. The PCR reactions were performed in a Perkin Elmer (Norwalk, CT) 9700 ThermalCycler with the following settings: 94°C for 10 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 49-59°C (depending on the amplicon), 1 min at 72°C, and one cycle for 7 min at 72°C. The PCR product were denatured for 5 min at 95°C and then slowly cooled to permit re-annealing and formation of homoduplexes and heteroduplexes. The PCR products were then stored at 4°C until DHPLC analysis.

DHPLC Analysis

DHPLC analysis was performed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) using buffer gradient and temperature conditions calculated using WAVEmaker software, (Version 3.3, Transgenomic) designed specifically for this purpose. An example of a wild-type and corresponding mutant chromatogram and base pattern alteration is illustrated in Figure 1.

For DNA samples displaying aberrant DHPLC chromatograms, the PCR was repeated using the same DNA and the forward and reverse DNA sequences of these PCR products was determined using an ABI PRISM 377 DNA Sequencer (Foster City, CA). The primers used for sequencing were the forward and reverse PCR primers.

Statistical Analysis

Differences in proportions were derived using the Pearson chi square model (40).

RESULTS

6 patients were found to have 8 novel *ATM* misense mutations and 1 rare *ATM* genetic variant. The specific location of these genetic alterations and their associated amino acid substitutions are listed in Table 3. The misense mutations were classified as non-conservative, conservative, or synonymous depending upon the type of amino acid substitution. 3 patients contained more than one novel or rare mutation.

The median follow up was 3.2 years (range: 1.3 – 10.3 years). All patients were found to have experienced at least a grade 1 acute skin reaction. 23 patients displayed RTOG/EORTC grade 2/3 patchy and/or confluent moist desquamation. 17 patients (37%) experienced a grade 1-4 late effect and 13 (28%) patients were found to have grade 2-4 late effects. Grade 1 late effects was not considered in the analysis as the descriptive criteria are minimal and subject to increased variability. The clinical descriptions of the 13 patients with significant late effects (grade 2-4) are shown in Table 4. 7 patients were found to have cutaneous telangiectasias. 4 were at the scar only. 5 patients experienced significant persistent hyperpigmentation and underlying fibrosis. One patient developed persistent breast pain and required a modified radical mastectomy for pain relief and developed subsequent soft tissue necrosis one year after radiation therapy.

Remarkably, all 3 patients (100%) who developed a grade 3-4 subcutaneous late effect, possessed *ATM* mutations. Of additional importance is

that each of these patients actually was found to harbor two *ATM* mutations. In contrast, only 7% (3/43) of patients who did not develop a grade 3-4 subcutaneous late response were found to be *ATM* carriers and in all of these cases only one mutation was detected.

A*TM* heterozygosity did not predict for a significant increase in acute effects. 4/23 (17%) patients with grade 2/3 moist desquamation had a mutation compared to 2/23 (9%) patients without evidence of acute morbidity (p=0.04)

The effects of moist desquamation, diabetes, tobacco use (smoker vs non-smoker), use of chemotherapy, total dose, and use of an electron boost were examined separately in relation to late effects and no independent variable achieved statistical significance (Table 5).

DISCUSSION

It has been hypothesized that *ATM* heterozygotes may exhibit enhanced radiosensitivity and be at an increased risk for radiation-induced normal tissue toxicity. However, no statistically significant correlation between *ATM* mutation status and normal tissue effects has been reported (20-24). To our knowledge, aside from case reports, this represents the first study that suggests such an association exists in the female breast cancer population.

Several studies have been performed in an attempt to identify a correlation between *ATM* heterozygosity and radiation-induced complications in breast cancer patients. Two of these studies (21, 22) may be discounted because they used PTT for mutation detection which is only capable of detecting protein truncation mutations. It appears, however, that the vast majority of alterations identified in cancer patients are missense mutations which would remain undetected by PTT (25). In a separate study (20), 16 breast cancer patients with excessive normal tissue changes were screened and no *ATM* mutations were detected. However, this study focused on patients with acute reactions and we have found a correlation only with late effects.

Another project (23) screened 11 breast cancer patients (along with 9 other patients exhibiting a variety of cancers) who developed RTOG grade level 3/4 complications and also found no *ATM* mutations. A plausible explanation for the negative findings of this study is that all of the grade 3/4 level complications in the breast cancer patients were either acute or skin reactions. None were

grade 3/4 level subcutaneous responses that we have found to correlate with *ATM* mutations. Weisberg et al. (24) obtained the medical records for breast cancer patients treated with radiotherapy who were blood relatives of AT patients and known to be carriers of an *ATM* mutation either through genetic marker analysis or having a child with AT. Eight of these patients exhibited an RTOG grade 2 acute response and two developed a grade 2 late reaction. However, this study is limited due to its small sample size and potential inaccuracies as questionnaires to the treating physicians or there successors were used at least in part to ascertain the nature of the radiation reactions rather than direct observation by the physician.

In contrast, Varghese et al. published a case report (19) of a patient who experienced severe skin and subcutaneous tissue late effects following conventional radiation therapy in the adjuvant setting for breast cancer. This patient was found to be an *ATM* heterozygote. Cellular assays demonstrated that cells from this patient had a radiosensitivity between the values for normal cells and those from patients with AT. Hall et al. (41) recently documented enhanced radiosensitivity in prostate cancer patients found to have excessive morbidity after external beam radiation therapy. Specifically, 18% of patients with a late effect possessed an *ATM* mutation compared to no mutations in their matched controls.

The mutation screening technique used in this study was denaturing high performance liquid chromatography (DHPLC). DHPLC is a robust technique that can be used to screen any gene in a large population for single nucleotide

substitutions, as well as small deletions and insertions (28-36). DNA sequence alterations can be detected in DNA fragments by fractionation of heteroduplexes on ion-pair reverse-phase columns. The advantage of DHPLC is that it enables the rapid, sensitive and accurate identification of polymorphisms and mutations in an automated fashion. Although this is a relatively new technique, it has already been used to screen a variety of genes. Of greatest importance for this study was the evidence that DHPLC possessed a sensitivity and specificity for mutation detection approaching 100% and that this approach possessed greater sensitivity than gel-based assays (28-36).

It is interesting to note that all 4 of the *ATM* mutations detected in patients exhibiting RTOG/EORTC grade 2-4 late responses were subcutaneous late normal tissue changes. There were no mutations in the patients with late cutaneous telangiectasias. The majority of the telangiectasias were only at the site of the surgical excision and were absent from the remaining breast surface. More importantly, 6/7 of these patients experienced patchy and/or moist desquamation that may account for subsequent development of telangiectasias. *ATM* mutations may render the subcutaneous tissues vulnerable to fibrotic changes as a result of inadequate repair in response to radiation therapy and loss of cellular integrity. There was only one case of a grade 4 reaction manifest by soft tissue necrosis and, of importance, this patient had two novel genetic alterations. She required hyperbaric oxygen therapy to which she responded with adequate wound healing.

It might be suggested that only the non-conservative missense mutations detected in this study be included in the data analysis as it could be postulated that only this class of missense mutation, which causes a significant change in the amino acid encoded by the *ATM* gene, would be of importance. Although this may true in many instances, not all substitutions have pathologic significance and may depend upon the location of the alteration within the protein and whether it affects important functional domains (42,43). Conversely, it might be assumed that non-conservative and synonymous mutations would have little or no impact upon protein function. However, this assumption may prove incorrect as some of these genetic alterations cause critical changes in protein function such as by inducing exon skipping (44,45). Additionally, the genetic alteration of greatest importance may be the presence of two *ATM* mutations, particularly if they are on opposite chromosomes. In these cases, as seen for patients manifesting grade 3-4 subcutaneous late responses, the two mutations may cause a mild enough change in the ATM protein so that the patient does not develop the disorder AT. However, the protein changes may be of sufficient importance, particularly if there are alterations in both *ATM* copies, that the patient will be more susceptible for the development of severe late sequelae. Therefore, the best way to examine whether a particular mutation or set of mutations affect protein activity is to perform functional assays. Such experiments are planned for future studies with cell lines derived from these patients as the scope of this work is beyond the current study. Hence, for the

purpose of this report, all missense mutations have been included in the analysis.

This study is too small to serve as the basis for therapeutic recommendations. However, our finding that 100% of breast cancer patients developed grade 3-4 level subcutaneous late responses following radiotherapy is of sufficient importance to necessitate a larger, more definitive study. If an expanded project confirms these findings, then breast cancer patients found to be carriers of *ATM* mutations may be better served with primary surgical resection rather than breast conservation management as late RTOG/EORTC grade 3-4 morbidity can lead to poor cosmesis and patient discomfort. Alternatively, these patients may be ideal candidates for dose reduction trials as *ATM* germline mutations should be present in both normal tissue and tumor cells, thus providing local control rates at reduced doses. DHPLC screening may be appropriate for newly diagnosed breast cancer patients to identify this potentially radiosensitive cohort, but it is premature to suggest implementation of *ATM* screening at the present time.

CONCLUSION

ATM heterozygosity in the female breast cancer population predicts for an increase in subcutaneous tissue effects following radiation therapy. DHPLC is effective for the identification of this radiosensitive cohort. These results suggest that *ATM* heterozygosity may be a relative contraindication to breast conservation management using conventional doses. Alternatively, germline

ATM mutation carriers may prove to be ideal candidates for radiation dose reduction trials. A larger study is required to confirm these findings.

ACKNOWLEDGEMENTS

Support from the Mount Sinai Ruttenberg Cancer Center is gratefully acknowledged which enabled purchase of the WAVE Nucleic Acid Fragment Analysis System.

REFERENCES

1. Syllaba K, Henner K. Contribution a l'independence de l'athetose double idiopathique et congenitale. Atteinte familiale, syndrome dystrophique, signe de reseau vasculaire conjonctival, integrite psychique. *Review of Neurology* 1926;1:541-562.

2. Sedgwick RP, Boder E. Ataxia-telangiectasia. In: Vianney De Jong JMB, editor. Hereditary and Spinocerebellar Atrophies. Amsterdam: Elsevier, Inc; 1991. p. 347-423.

3. Morgan JL, Holcomb TM, Morrissey RW. Radiation reaction in ataxia-telangiectasia. *Am J Dis Child* 1968;116:557-558.

4. Swift M, Morrell D, Cromartie E, *et al.* The incidence and gene frequency of ataxia-telangiectasia in the United States. *Am J Hum Genet* 1986;39:573-583.

5. Gatti RA, Tward A, Concannon P. Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations. *Mol Genet Metab* 1999;68:419-423.

6. Dahlberg W, Little JB. Response of dermal fibroblast cultures from patients with unusually severe responses to radiotherapy and from ataxia telangiectasia heterozygotes to fractional radiation. *Clin Can Res* 1995;1:785-790.

7. Cole J, Arlett CF, Green MH, *et al.* Comparative human cellular radiosensitivity: II. The survival following gamma-irradiation of unstimulated (Go) T-lymphocyte lines, lymphoblastoid cell lines and fibroblasts from normal donors, from ataxia telangiectasia patients and from ataxia telangiectasia heterozygotes. *Int J Rad Biol* 1988;54:929-942.

8. Swift M, Morell D, Massey RB, *et al.* Incidence of cancer in 161 families affected by ataxia-telangiectasia. *NEJM* 1991;325(26):1831-1836.

9. Atencio DP, Iannuzzi CM, Rosenstein BS. Screening breast cancer patients for ATM mutations and polymorphisms using denaturing high performance liquid chromatography. *Environ Mol Mutagen* (accepted for publication).

10. Watters D, Khanna KK, Beamish H, *et al.* Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. *Oncogene* 1997;14:1911-1921.

11. Canman CE, Lim DS, Cimprich KA, *et al.* Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* 1998;281:1677-1679.

12. Sarkaria JN, Tibbetts RS, Busby EC, *et al.* Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res* 1998;58:4375-4382.

13. Banin S, Moyal L, Shieh S, *et al.* Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998;281:1674-1677.

14. Gately DP, Hittle JC, Chan GK, *et al.* Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity. *Mol Biol Cell* 1998;9:2361-2374.

15. Martinho R, Lindsay HD, Flaggs G, *et al.* 1998. Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. EMBO J. 17:7239-7249.

16. Matsuoka S, Rotman G, Ogawa A, *et al.* Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci USA* 2000;97:10389-10394.

17. Khanna KK. Cancer risk and the ATM gene: a continuing debate. *J NCI* 2000;82(10)795-802.

18. Meyn SM. Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene. *Clin Genet* 1999;55:289-304.

19. Varghese S, Schmidt-Ullrich RK, Dritschilo A, *et al.* Enhanced radiation late effects and cellular radiation sensitivity in an ATM heterozygous breast cancer patient. *Rad Onc Inves* 1999;7:231-237.

20. Appleby JM, Barber JB, Levine E, *et al.* Absence of mutations in the ATM gene in breast cancer patients with severe responses to radiotherapy. *BJ Cancer* 1997;76(12):1546-1549.

21. Ramsay J, Birrell G, Lavin M. Testing for mutations of the ataxia telangiectasia gene in radiosensitive breast cancer patients. *Rad Onc* 1998;47:125-128.

22. Clarke RA, Goozee GR, Birrell G, *et al.* Absence of ATM truncations in patients with severe acute radiation reactions. *IJROBP* 1998;41(5):1021-1027.

23. Oppitz U, Bernthaler U, Schindler D, *et al.* Sequence analysis of the ATM gene in 20 patients with RTOG grade 3 or 4 acute and/or late tissue radiation side effects. *IJROBP* 1999;44(5):981-988.

24. Weisberg JB, Huang DD, Swift M. Radiosensitivity of normal tissues in ataxia-telangiectasia heterozygotes. *IJROBP* 1998;42(5):1133-1136.

25. Concannon P, Gatti RA. Diversity of ATM gene mutations detected in patients with ataxia-telangiectasia. *Hum Mutat* 1997;10:100-107.

26. Gilad S, Khosravi R, Shkedy D, *et al.* Predominance of null mutations in ataxia- telangiectasia. *Hum Mol Genet* 1996;5:433-439.

27. Telatar M, Teraoka S, Wang Z, *et al.* Ataxia-telangiectasia: identification and detection of founder-effect mutations in the ATM gene in ethnic populations. *Am J Hum Genet* 1998;62:86-97.

28. Gross E, Arnold N, Goette J, *et al.* A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. *Hum Genet* 1999;105:72-78.

29. Gross E, Arnold N, Pfeifer K, *et al.* Identification of specific BRCA1 and BRCA2 variants by DHPLC. *Hum Mutat* 2000;16:345-353.

30. Huber CG, Oefner PJ, Bonn GK. High-resolution liquid chromatography of oligonucleotides on nonporous alkylated styrene-divinylbenzene copolymers. *Anal Biochem* 1993;212:351-358.

31. Huber CG, Oefner PJ, Bonn GK. Rapid and accurate sizing of DNA fragments by ion-pair chromatography on alkylated nonporous polystyrenedivinylbenzene particles. *Anal Chem* 1995;67:578-585.

32. Kuklin A, Munson K, Gjerde D *et al.* Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system. *Genet Test* 1997;1:201-206.

33. Arnold N, Gross E, Schwarz-Boeger U, *et al.* A highly sensitive, fast, and economical technique for mutation analysis in hereditary breast and ovarian cancers. *Hum Mutat* 1999;14:333-339.

34. Liu W, Smith DI, Rechtzigel KJ, *et al.* Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res* 1998;26:1396-1400.

35. O'Donovan MC, Oefner PJ, Roberts SC, *et al.* Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. *Genomics* 1998;52:44-49.

36. Wagner T, Stoppa-Lyonnet D, Fleischmann E, *et al.* Denaturing high-performance liquid chromatography detects reliably BRCA1 and BRCA2 mutations. *Genomics* 1999;62:369-376.

37. Fleming ID, Cooper JS, Henson DE, Hutter RVP, Kennedy BJ, Murphy GP. American Joint Committee on Cancer. Cancer Staging Manual. 5th ed. Philadelphia (PA):Lippincott-Raven; 1997.

38. Cox JD, Stetz J, Pajak TF. Toxicity criteria of the Radiation Therapy Oncology group (RTOG) and the European Organization for Reasearch and Treatment of Cancer (EORTC). *IJROBP* 1995;31(5):1341-1346.

39. Teraoka SN, Telatar M, Becker-Catania S, *et al.* Splicing defects in the ataxia-telangiectasia gene, ATM: Underlying mutations and consequences. *Am J Hum Genet* 1999;64:1617-1631.

40. Feinberg, SE. The analysis of cross-classified categorical data, 2nd ed. Cambridge, MA: MIT Press; 1980.

41. Hall EJ, Schiff PB, Hanks GE, *et al.* A preliminary report: frequency of AT heterozygotes among prostate cancer patients with severe late responses to radiation therapy. *Ca J Sci Amer* 1998;4(6):385-389.

42. Lavin MF, Khanna KK, Beamish H, *et al.* Relationship of the *ATM* gene (mutated in ataxia-telangiectasia) to phosphatidylinositol 3-kinase. *Trends Biochem Sci* 1995;20:382-382.

43. Savitsky K, Bar-Shira A, Gilad S, *et al.* A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 1995;268:1749-1753.

44. Richard I, Beckmann JS. 1995. How neutral are synonymous codon mutations? *Nat Genet* 1995;2:1719-1721.

45. Liu W, Qian C and Francke U. Silent mutation induces exon skipping of fibrillin-1 gene in Marfan syndrome. Nat Genet 1997;16:328-329.

Table 1. Breast Cancer Patient Demographics and Tumor Properties

Patient demographics/ Tumor characteristics	# of patients	%
Age (years):		
Under 40	2	4
40-59	16	35
60-80	30	61
Ethnicity:		
Caucasian	22	48
Hispanic	18	39
Black	5	11
Asian	1	2
Menopausal status:		
Pre	15	33
Post	31	67
AJCC Stage:		
DCIS	5	11
I	29	63
II	12	26
Histology:		
DCIS	5	11
Invasive Ductal	38	83
Invasive Lobular	3	6
Diabetes:		
Yes	5	11
No	41	89
History of smoking:		
Yes	16	35
No	30	65

Table 2: RTOG/EORTC Acute and Late Morbidity Schema

Grade	Acute Skin Criteria
1	Faint or dull erythema/epilation/dry desquamation/decreased sweating
2	Tender or bright erythema/patchy moist desquamation/moderate edema
3	Confluent moist desquamation other than skin folds/pitting edema
4	Ulceration/ hemorrhage/necrosis

Grade	Late Skin Criteria	Late Subcutaneous Tissue Criteria
1	Slight atrophy/pigmentation change/some hair loss	Slight induration (fibrosis) and loss of subcutaneous fat
2	Patch atrophy/moderate telangiectasia/total hair loss	Moderate fibrosis but asymptomatic/slight field contracture/ <10% linear reduction
3	Marked atrophy/gross telangiectasia	Severe induration and loss of subcutaneous tissue/field contracture > 10% linear measurement
4	Ulceration	Necrosis
5	Death	Death

*Adapted from Cox et al. IJROBP 31(5)1341-1346, 1995.

Table 3. Mutations and Polymorphisms Identified in Breast Cancer Patients Using DHPLC

Non-conservative Missense				
Patient Number	Location	Exon	Codon Number	Amino Acid Change
30	2119T→C	15	707	ser→pro
45	4138C→T	30	1380	his→tyr
45	4400A→G	31	1467	asp→gly
49	2362A→C	17	788	ser→arg
Conservative Missense				
Patient Number	Location	Exon	Codon Number	Amino Acid Change
34	7397C→T	52	2466	ala→val
49	6088A→G	43	2030	ile→val
Synonymous				
Patient Number	Location	Exon	Codon Number	Amino Acid Change
12	5793T→C	41	1931	ala→ala
21	1176C→G	11	392	gly→gly
34	735C→T	9	245	val→val

Table 4. Thirteen Patients with RTOG/EORTC Grade 2-4 Late Normal Tissue Effects

Patient #	*ATM* Mutation	Acute Skin Reaction*	Late Skin/Subcutaneous Tissue Reaction
21	+	grade 2 patchy MD	grade 2 fibrosis
45	+	grade 1 erythema	grade 3 fibrosis
49	+	grade 2 patchy MD	grade 3 fibrosis
34	+	grade 3 confluent MD	grade 4 soft tissue necrosis
5	-	grade 2 patchy MD	grade 2 telangiectasia at scar
7	-	grade 1 erythema	grade 2 telangiectasia at scar
23	-	grade 3 confluent MD	grade 2 diffuse telangiectasias
46	-	grade 2 patchy MD	grade 2 telangiectasia at scar
40	-	grade 3 confluent MD	grade 3 diffuse telangiectasias
11	-	grade 2 patchy MD	grade 2 telangiectasia at scar
39	-	grade 1 erythema	grade 2 fibrosis
13	-	grade 2 patchy MD	grade 2 fibrosis
25	-	grade 3 confluent MD	grade 3 diffuse telangiectasias

*MD=moist desquamation

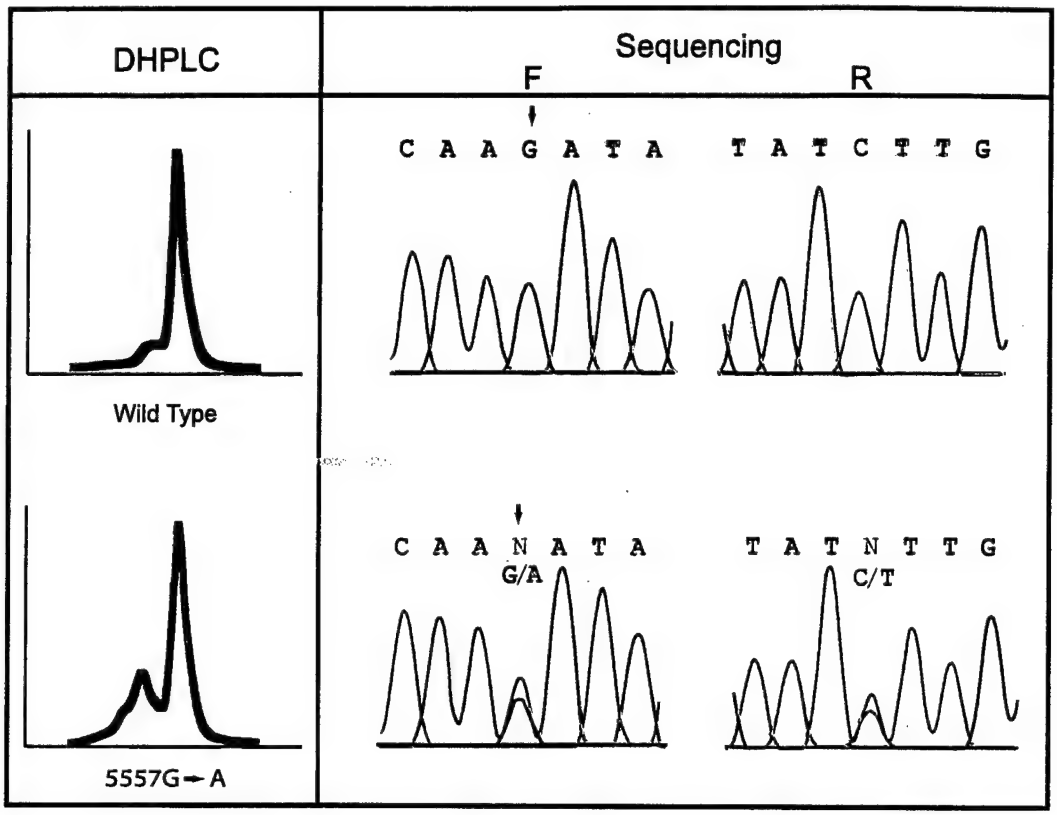
Table 5. Univariate Analysis of variables that may predict for RTOG/EORTC Grade 2-4 Late Effects

Variable	p value*
Total dose	
>50 Gy	0.4
>60 Gy	0.4
Diabetes	0.09
Smoking	0.3
Chemotherapy	0.7
Electron boost	0.9
Acute effects (grade 2/3)	0.3
ATM mutation	0.02

*Pearson chi square model

FIGURE LEGEND

Figure 1. A representative example of a wild-type pattern and a genetic variant DHPLC chromatogram. The double peak is indicative of a change in the base pair sequence and subsequent amino acid substitution.


DHPLC
Sequencing
F
R
C A A G A T A
T A T C T T G
Wild Type
C A A N A T A
G/A
T A T N T T G
C/T
5557G→A

Revised 6/13/01

Environmental and Molecular Mutagenesis

Screening Breast Cancer Patients for *ATM* Mutations and Polymorphisms Using Denaturing High Performance Liquid Chromatography

David P. Atencio,[1] Christopher M. Iannuzzi,[1] Sheryl Green, [1] Richard G. Stock, [1] Jonine L. Bernstein[2] and Barry S. Rosenstein[1,2, 3, 4]

[1]Departments of Radiation Oncology, [2]Community & Preventive Medicine and [3]Dermatology, Mount Sinai School of Medicine, New York, New York

[4]Department of Radiation Oncology, New York University School of Medicine, New York, New York

Grant sponsor: Breast Cancer Research Fund of the Department of Defense; Grant Number: DAMD17-97-1-7208.

*Correspondence to: Barry S. Rosenstein, Department of Radiation Oncology, Box 1236, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: barry.rosenstein@mssm.edu

All 62 coding exons of the *ATM* gene, along with 10-20 bases of the intronic region flanking each exon, were screened for DNA base sequence alterations using denaturing high performance liquid chromatography (DHPLC) in a series of 52 breast cancer patients. Six (12%) of these patients exhibited a total of 8 different novel germline mutations that do not represent common polymorphisms. Of these, 3 patients possessed 4 nonconservative missense mutations while 2 conservative missense and 2 synonymous mutations were detected in the other 3 patients. In addition, 43 patients were found to have a total of 141 DNA sequence variations representing 21 different common polymorphisms and rare variants. An analysis of the relationship between the presence of a novel *ATM* mutation and either patient demographics or tumor properties demonstrated a significant difference between African Americans (3/7 = 43%) and other ethnic groups (3/45 = 7%, $p = 0.026$). None of the other characteristics examined was found to be related to mutation status.

Key words: African American; *ATM*; breast cancer; DHPLC; mutations; polymorphisms

INTRODUCTION

ATM is the gene whose mutation results in the disease ataxia telangiectasia (AT). This is an autosomal recessive disease first described by Syllaba and Henner [1926] and characterized by a pleiotropic phenotype encompassing multiple organ systems. Classically, the disorder is associated with progressive cerebellar degeneration, immunodeficiency, oculotaneous telangiectasia, and increased sensitivity to ionizing radiation [Sedgwick and Boder, 1991]. The gene for this disease is located on chromosome 11q22-23 [Gatti et al., 1988; Lange et al., 1995] and was cloned and sequenced by Savitsky et al. [1995]. It is 157 kbp and contains 66 coding exons with the start and stop codons located within exons 4 and 65, respectively. The ATM protein is approximately 370 kDa in size [Chen and Lee, 1996] and is a member of the phosphatidylinositol 3 kinase superfamily [Savitsky et al., 1995]. However, evidence has been obtained indicating that it functions primarily as a protein kinase [Brunn et al., 1996; Banin et al., 1998; Canman et al., 1998; Sarkaria et al., 1998].

The ATM gene product has been the subject of intensive investigation and appears to play a central role in cell cycle regulation and genomic stability. It is primarily located within the nucleus of dividing cells [Watters et al., 1997] and displays increased phosphorylation activity of effector substrates in response to double strand DNA damage that will delay the cell cycle at critical checkpoints to

allow for DNA repair prior to replication. These downstream effects are critical to the genetic integrity of the cell. The G1/S checkpoint has been well characterized and phosphorylation of p53 [Banin et al., 1998; Canman et al., 1998] by ATM is a necessary step towards cell cycle arrest in response to a DNA insult. There is also mounting evidence that ATM plays a role in other cell cycle checkpoints [reviewed by Khanna, 2000]. S phase regulation appears to be mediated through ATM phosphorylation of the RPA protein [Gately et al., 1998] and the G2/M checkpoint is controlled, at least in part, through phosphorylation of Chk1 [reviewed by Lavin, 1999; Martinho et al., 1998] and Chk2 [Matsuoka et al., 2000]. Other important substrates for this kinase include the early response gene products c-Abl [Shafman et al., 1997; Kharbanda et al., 1996] and NF-κB, [Lee et al., 1998; Piret et al., 1999; Li et al. 2000] as well as BRCA1 [Gatei et al., 2000; Cortez et al., 1999] all of which are involved in DNA surveillance and repair (both homologous and non-homologous) processes [Morrison et al., 2000; Kanaar et al., 1998]. Cells lacking such activity have demonstrated a predilection towards increased mutagenesis and chromosomal aberrations.

Symptoms of the disease AT are manifested only for individuals possessing two mutated copies of the *ATM* gene. The majority of *ATM* mutations detected in AT patients are truncating mutations [Gilad et al., 1996; Concannon and Gatti 1997; Telatar et al., 1998]. Although AT is a relatively rare disease with an incidence of approximately 1/100,000 [Swift et al. 1986; Woods et al. 1990], it

has been estimated that about 1% of the population are carriers of a single mutated copy of the gene [Swift et al., 1986]. *ATM* heterozygosity does not cause the disease, but it has been hypothesized that *ATM* carriers possessing missense mutations are more susceptible to various forms of cancer [Gatti et al., 1999].

A series of studies has been performed to determine whether the percentage of *ATM* carriers in groups of patients with several different forms of cancer is greater than the 1% of the general population estimated to be *ATM* heterozygotes. Conflicting results have been found for breast cancer [Athma et al., 1996; Vorechovsky et al., 1996a; Fitzgerald et al., 1997; Bay et al., 1998; Izatt et al., 1999; Broeks et al., 2000; Kovalev et al., 2000] while an elevated *ATM* mutation frequency has been reported for mantle cell lymphoma [Schaffner et al., 2000; Stilgenbauer et al., 2000], B-cell lymphocytic leukemia [Stankovic et al., 1999], adult acute lymphoblastic leukemia [Haidar et al., 2000] and sporadic T-cell leukemia [Vorechovsky et al., 1997]. There are, however, significant limitations to these studies in that they were all performed with less than optimal screening technologies. The screenings using the protein truncation test (PTT) [Roest et al., 1993] were only capable of identifying truncating mutations that are causative of AT. In contrast, missense mutations are apparently associated with cancer susceptibility and these mutations went undetected in the PTT work. In addition, studies were performed using gel-based assays such as single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) that are

also limited in their mutation detection capability [Choy et al., 1999; Jones et al., 1999; Wagner et al., 1999a; Gross et al., 1999, 2000; Benit et al., 2000].

In contrast, the technique used for mutation detection in this study was DHPLC [Huber et al., 1993; Huber et al., 1995; Kuklin et al., 1997; Oefner and Underhill, 1998]. DHPLC is a robust technique that can be used to screen any gene in a large population for single nucleotide substitutions, as well as small deletions and insertions. DNA sequence alterations can be detected in DNA fragments by fractionation of heteroduplexes on ion-pair reverse-phase columns. The advantage of DHPLC is that it enables the rapid, sensitive and accurate identification of polymorphisms and mutations in an automated fashion. Although this is a relatively new technique, it has already been used to screen a variety of genes [Wagner et al., 1998; Wagner et al., 1999b; Yokomizo et al., 1998; Skopek et al., 1999; Jin et al., 1999; Mai et al., 1998; Giordano et al., 1999; Underhill et al., 1997; McCallum et al., 2000; Schriml et al., 2000].

Briefly, the basis of DHPLC used for mutation detection is that cells heterozygous for a DNA sequence alteration have a 1:1 ratio of DNA possessing the alternate alleles. Heating the polymerase chain reaction (PCR) products to single-stranded form at 95°C and cooling slowly to permit hybridization results in the formation of a mixture of heteroduplexes and homoduplexes. DHPLC analysis is performed at a temperature sufficient to partially denature the DNA heteroduplexes,

which are resolved from homoduplexes using ion-pair reversed phase liquid chromatography. Therefore, if a DNA sample does not possess a base sequence alteration, only homoduplexes will form and only one peak should appear on the DHPLC chromatogram representing the homogenous nature of the fragment. A sample that contains a heterozygous sequence alteration will display 2, 3 or 4 peaks reflecting the two homoduplex and two heteroduplex populations. Of greatest importance for this project is the evidence that DHPLC possesses a sensitivity and specificity for mutation detection approaching 100% and that this approach is superior to gel-based assays [O'Donovan et al., 1998; Liu et al., 1998; Arnold et al., 1999; Choy et al., 1999; Jones et al., 1999; Wagner et al., 1999a; Gross et al., 1999, 2000; Nickerson et al., 2000; Taniguchi et al., 2000].

MATERIALS AND METHODS

Patients

Peripheral venous blood samples (3 ml) were obtained from a hospital-based series of 52 unselected patients diagnosed with primary breast cancer treated with conservative surgery and adjuvant radiation therapy with or without chemotherapy between 1992 and 1997. The nature of the project was discussed with each patient and signed informed consent was obtained prior to obtaining each blood sample. Patient demographics and tumor properties were obtained by chart review and are presented in Table I.

DNA Isolation

Lymphocytes were isolated by Ficoll-Paque separation. 1.5 ml Ficoll-Paque was added to 3 ml of blood and centrifuged at 12000 rpm for 20 min at room temperature. The lymphocytes layer was collected, washed with 10 ml PBS and centrifuged for 10 min, room temperature, at 12,000 rpm.

To isolate DNA from the lymphocytes, 1 ml DNA STAT-60 (Tel-Test, Friendswood, TX) was added to whole blood, followed by addition of 200 µl of chloroform and centrifuged at 4°C for 15 min at 12,000 rpm. The aqueous layer

was transferred to a fresh 1.5 ml tube and the extraction procedure repeated. Following an additional chloroform extraction, 500 µl of isopropanol was added to the supernatant. The tube was held at -20°C overnight and centrifuged 10 min at 4°C. The supernatant was removed and the pellet washed with 200 µl of 70% ethanol. After centrifugation at 4°C, 12,000 rpm, for 8 min, the supernatant was removed and the ethanol wash/precipitation was repeated. The supernatant was removed, the pellet air-dried and 150 µl H_2O and 1.5 µl TE buffer was added.

PCR

50 µl PCR reactions were performed to amplify each of the 62 *ATM* exons that comprise the translated portion of the *ATM* gene using primers previously described [Teraoka et al. 1999]. Each PCR reaction contained 100 ng of genomic DNA, 200 µM of each dNTP, 250 nM primers, 2.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA) in Qiagen 10X buffer diluted to 1X. RNase free water was then added to a total volume of 50 µl. The PCR reactions were performed in a Perkin Elmer (Norwalk, CT) 9700 ThermalCycler with the following settings: 94°C for 10 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 49-59°C (depending on the amplicon), 1 min at 72°C, and one cycle for 7 min at 72°C. The PCR products were denatured for 5 min at 95°C and then slowly cooled to permit re-annealing and formation of homoduplexes and heteroduplexes. The PCR products were stored at 4°C until DHPLC analysis.

DHPLC Analysis

DHPLC analysis was performed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) using buffer gradient and temperature conditions calculated using WAVEmaker software, (Version 3.3, Transgenomic) designed specifically for this purpose.

For DNA samples displaying aberrant DHPLC chromatograms, the PCR was repeated and the forward and reverse DNA sequences of these PCR products determined using an ABI 377 DNA Sequencer (Foster City, CA). The primers used for sequencing were the forward and reverse PCR primers.

Statistical Analysis

Fisher's Exact Test was used to examine the relationship between patient demographics or tumor properties and the presence of an *ATM* mutation. P-values less than 0.05 (2-sided test) were considered significant [Kendall and Stuart, 1979; Mehta and Patel, 1983]. Ninety-five percent confidence intervals were constructed using the Normal approximation to the binomial distribution.

RESULTS

DNA was isolated from peripheral lymphocytes obtained from a series of 52 breast cancer patients whose demographics and tumor properties are outlined in Table I. Each of the 62 translated *ATM* exons, along with 10-20 bases of the adjoining intron, was amplified for every patient and screened using DHPLC. The PCR product was sequenced for every instance in which an aberrant chromatogram was produced. A total of 151 chromatogram profiles were produced that differed from the profiles obtained using wild type DNA as the PCR template. Representative examples of aberrant chromatograms with multiple peaks are shown in Fig. 1. The forward and reverse sequences of the DNA regions of interest were determined for these patients. The sequencing results confirmed that 149 of the 151 DNA fragments possessed DNA alterations (Table II) yielding a 1% false positive rate for DHPLC in this study.

It should be noted that, theoretically, only one peak should be observed on the chromatograms produced using wild type DNA. However, as can be seen for the two exons whose DHPLC profiles are shown in Fig. 1, secondary peaks are often observed. Presumably, this is due both to primer length disparity and/or non-specific amplification. However, this does not detract from the sensitivity of the assay as the profiles produced from each sample not possessing a mutation are nearly identical. Hence, any sample possessing a DNA sequence alteration can be

identified as even a slight deviation from the DHPLC profile produced from wild type cells signals heteroduplex formation. This is true even when the change is somewhat subtle as exemplified for the heteroduplexes containing the 2362A→C mutation in exon 17 (Fig. 1).

Of the 149 DNA sequence alterations identified, 4 represent nonconservative missense mutations and are predicted to cause the substitution of one amino acid by another with a dissimilar side chain that could affect the function of the ATM protein. That is, the 2119C→T would substitute a hydrophobic in place of a polar amino acid, the 4400A→G would cause replacement of a neutral for an acidic amino acid, the 4138C→T results in encoding a neutral in place of a basic amino acid and the 2362A→C would switch a basic for a neutral acid amino acid. All 4 of these mutations were novel (Table II). Two patients were found to have conservative missense mutations, not previously identified as common polymorphisms, in which the encoded amino acid would be replaced by one that is chemically similar. In addition, two women possessed novel synonymous mutations in which the codon found in wild type DNA was replaced with one that encodes the same amino acid. The remaining DNA sequence alterations all represented common polymorphisms and rare variants with frequencies found in this study consistent with other reports. None of the intronic alterations were located in splice site junctions.

With respect to the patient demographics and tumor properties listed in Table I, one ethnic group was found to be significantly related to the presence of an *ATM* mutation. 3 of 7 (43%) African American patients (12, 21 and 45) had a novel *ATM* mutation compared with 3 of 45 (7%) patients (30, 34 and 49) in other ethnic groups ($p = 0.026$).

DISCUSSION

A series of 52 breast cancer patients was screened for DNA sequence variations in the *ATM* gene. A total of 8 mutations that do not represent common polymorphisms were detected in the coding regions of this gene for 6 patients. Of particular importance, 3 patients, representing roughly 6% of the patient population examined, possessed nonconservative missense mutations. Using this result, the 95% confidence interval for breast cancer patients possessing nonconservative *ATM* mutations was calculated to be 0-12%. An additional 3 women possessed either conservative missense or synonymous mutations. Although it might be predicted that these would generally be neutral mutations, they could still have functional significance. For example, it has been shown that even synonymous substitutions can cause substantial changes to encoded proteins by inducing exon skipping [Liu et al., 1997; Richard and Beckmann, 1995]. In addition, it is interesting to note that no truncating mutations were detected. This finding is consistent with the hypothesis that this type of mutation is not associated with cancer susceptibility. Hence, the results of this study are supportive of the hypothesis that the frequency of breast cancer patients possessing *ATM* mutations is greater than the approximately 1% of the general population estimated to be *ATM* carriers. Despite the obvious limitations resulting from the relatively small sample size of this study, this report also provides the first evidence that *ATM* heterozygosity may be associated with breast cancer susceptibility for African

American women and so may serve as the basis for further investigation regarding *ATM* mutations among African American breast cancer patients.

Another important result of this study is the discovery of a person possessing two non-conservative *ATM* mutations, but manifesting no symptoms of the disease AT. To the best of our knowledge, this 75 year-old woman represents the first person ever to be identified possessing two mutated copies of the *ATM* gene but not exhibiting AT symptoms. The likely explanation for her lack of phenotypic manifestations of AT is that both alterations in her *ATM* gene are missense mutations, as opposed to the truncating mutations found in a high percentage of AT patients. Therefore, this person is likely capable of producing at least a partially functional ATM protein as opposed to the non-functional product generally encoded by the *ATM* gene in AT patients.

It is significant to note that 37 of the 52 patients that are the subject of this report were previously screened using a non-isotopic RNase cleavage assay (NIRCA), an alternate mutation detection assay [Drumea et al 2000]. In that study, base sequence variations were detected in only 8 of the 37 patients and all represented the common 5557G→A polymorphism. Identification of intronic alterations was not possible in that project as cDNAs were examined. However, the results of the current study using DHPLC demonstrate that NIRCA failed to detect DNA sequence variations located in the exons of 8 of the 37 patients. This finding

demonstrates the superiority of DHPLC for mutation detection compared with NIRCA.

It has been estimated that the relative risk of breast cancer for *ATM* carriers is approximately 4-fold and that these women account for roughly 4% of all breast cancer cases [Easton, 1994]. This confers a clinically significant cancer risk making the susceptibility associated with this genetic alteration comparable to the breast cancer risk resulting from a mutation in either the *BRCA1* or *BRCA2* genes. Therefore, population screening for *ATM* mutations and vigilant surveillance of *ATM* carriers may prove to be appropriate. The issue of increased mammographic screening for *ATM* carriers is controversial [Gray, 1992; Werneke, 1997] and may be less than ideal given the possible enhanced sensitivity for radiation-induced breast cancer. A more appropriate mode of detection may be serial magnetic resonance imaging (MRI) of the breast, a modality that does not utilize ionizing radiation. There is evidence suggesting that this may represent an effective screening tool in high-risk patients [Tilanus-Linthorst et al., 2000]. We would further postulate that given the apparent overall increased breast cancer risk for *ATM* carriers, that population screening may be beneficial for detection of people susceptible to other forms of cancer. Through use of the powerful DHPLC mutation detection methodology, it is now feasible to examine large populations exhibiting

specific forms of cancer to determine the percentage of *ATM* carriers in these populations.

ACKNOWLEDGMENTS

I, Barry Rosenstein, am privileged to contribute to this *Festschrift* in honor of Dick Setlow. The stimulating atmosphere of Dick's laboratory, and daily lunch group conversations that I had the good fortune to have participated in during the time I was a postdoctoral fellow in Dick's laboratory, will always serve as an example of how a collection of scientists at various stages of their careers could ideally interact. The model Dick provided as an outstanding teacher, and subsequently as a life-long mentor, have shaped my career and relationship with my students and will undoubtedly continue to influence future generations of scientists.

REFERENCES

Arnold N, Gross E, Schwarz-Boeger U, Pfisterer J, Jonat W, Kiechle M. 1999. A highly sensitive, fast, and economical technique for mutation analysis in hereditary breast and ovarian cancers. Hum Mutat 14:333-339.

Athma P, Rappaport R, Swift M. 1996. Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer. Cancer Genet Cytogenet 92:130-134.

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281:1674-1677.

Bay JO, Grancho M, Pernin D, Presneau N, Rio P, Tchirkov A, Uhrhammer N, Verrelle P, Gatti RA, Bignon YJ. 1998. No evidence for constitutional ATM mutation in breast/gastric cancer families. Int J Oncol 12:1385-1390.

Benit P, Kara-Mostefa A, Berthelon M, Sengmany K, Munnich A, Bonnefont JP. 2000. Mutation analysis of the hamartin gene using denaturing high performance liquid chromatography. Hum Mutat 16: 417-421.

Broeks A, Urbanus JH, Floore AN, Dahler EC, Klijn JG, Rutgers EJ, Devilee P, Russell NS, van Leeuwen FE, van't Veer LJ. 2000. ATM-heterozygous germline mutations contribute to breast cancer- susceptibility. Am J Hum Genet 66: 494-500.

Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC, Abraham RT. 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J 15: 5256-5267.

Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. 1998. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281:1677-1679.

Castellvi-Bel S, Sheikhavandi S, Telatar M, Tai LQ, Hwang M, Wang Z, Yang Z, Cheng R, Gatti RA. 1999. New mutations, polymorphisms, and rare variants in the ATM gene detected by a novel SSCP strategy. Hum Mutat 14:156-162.

Chen G, Lee EYHP. 1996. The product of the ATM gene is a 370kDa nuclear phosphoprotein. J Biol Chem 271:33693-22697.

Choy YS, Dabora SL, Hall F, Ramesh V, Niida Y, Franz D, Kasprzyk-Obara J, Reeve MP, Kwiatkowski DJ. 1999. Superiority of denaturing high performance liquid chromatography over single-stranded conformation and conformation-

sensitive gel electrophoresis for mutation detection in TSC2. Ann Hum Genet 63:383-391.

Concannon P, Gatti RA. 1997. Diversity of ATM gene mutations detected in patients with ataxia-telangiectasia. Hum Mutat 10:100-107.

Cortez D, Wang Y, Qin J, Elledge SJ. 1999. Requirement of ATM-dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks. Science 286:1162-1166.

Drumea KC, Levine E, Bernstein J, Shank B, Green S, Kaplan E, Mandell L, Cropley J, Obropta J, Braccia I, Krupnick A, Rosenstein BS. 2000. ATM heterozygosity and breast cancer: screening of 37 breast cancer patients for ATM mutations using a non-isotopic RNase cleavage-based assay. Breast Cancer Research and Treatment 61:79-85.

Easton DF. 1994. Cancer risks in A-T heterozygotes. Int J Radiat Biol 66: S177-S182.

Ejima Y, Yang L, Sasaki MS. 2000. Aberrant splicing of the ATM gene associated with shortening of the intronic mononucleotide tract in human colon tumor cell lines: A novel mutation target of microsatellite instability. Int J Cancer 86:262-268.

Fitzgerald MG, Bean JM, Hegde SR, Unsal H, MacDonald DJ, Harkin DP, Finkelstein DM, Isselbacher KJ, Haber DA. 1997. Heterozygous ATM mutations do not contribute to early onset of breast cancer. Nat Genet 15:307-310.

Gatei M, Scott SP, Filippovitch I, Soronika N, Lavin MF, WeberB, Khanna KK. 2000. Role for ATM in DNA damage-induced phosphorylation of BRCA1. Cancer Res 60:3299-3304.

Gately DP, Hittle JC, Chan GK, Yen TJ. 1998. Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity. Mol Biol Cell 9:2361-2374.

Gatti RA, Berkel I, Boder E, Braedt G, Charmley P, Concannon P, Ersoy F, Foroud T, Jaspers NG, Lange K. 1988. Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. Nature 336:577-580.

Gatti RA, Tward A, Concannon P. 1999. Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations. Mol Genet Metab 68:419-423.

Gilad S, Khosravi R, Shkedy D, Uziel T, Ziv Y, Savitsky K, Rotman G, Smith S, Chessa L, Jorgensen TJ, Harnik R, Frydman M, Sanal O, Portnoi S, Goldwicz Z, Jaspers NG, Gatti RA, Lenoir G, Lavin MF, Tatsumi K, Wegner RD, Shiloh Y, Bar-Shira A. 1996. Predominance of null mutations in ataxia-telangiectasia. Hum Mol Genet 5:433-439.

Giordano M, Oefner PJ, Underhill PA, Cavalli-Sforza LL, Tosi R, Richiardi PM. 1999. Identification by denaturing high-performance liquid chromatography of numerous polymorphisms in a candidate region for multiple sclerosis susceptibility. Genomics 56:247-253.

Gray JE 1992. Increased incidence of cancer in a small subset of the population: a new obstacle to screening mammography? Radiology 185:285.

Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M. 1999. A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. Hum Genet 105:72-78.

Gross E, Arnold N, Pfeifer K, Bandick K, Kiechle M. 2000. Identification of specific BRCA1 and BRCA2 variants by DHPLC. Hum Mutat 16:345-353.

Hacia JG, Sun B, Hunt N, Edgemon K, Mosbrook D, Robbins C, Fodor SPA, Tagle DA, Collins FS. 1998. Strategies for mutational analysis of the large multiexon ATM gene using high-density oligonuleotide arrays. Genome Res 8:1245-1258.

Haidar MA, Kantarjian H, Manshouri T, Chang CY, O'Brien S, Freireich E, Keating M, Albitar M. 2000. ATM gene deletion in patients with adult acute lymphoblastic leukemia. Cancer 88:1057-1062.

Huber CG, Oefner PJ, Bonn GK. 1993. High-resolution liquid chromatography of oligonucleotides on nonporous alkylated styrene-divinylbenzene copolymers. Anal Biochem 212:351-358.

Huber CG, Oefner PJ, Bonn GK. 1995. Rapid and accurate sizing of DNA fragments by ion-pair chromatography on alkylated nonporous polystyrenedivinylbenzene particles. Anal Chem 67:578-585.

Izatt L, Greenman J, Hodgson S, Ellis D, Watts S, Scott G, Jacobs C, Liebmann R, Zvelebil MJ, Mathew C, Solomon E, 1999. Identification of germline missense mutations and rare allelic variants in the ATM gene in early-onset breast cancer. Genes Chromosomes Cancer 26:286-294.

Jin L, Underhill PA, Doctor V, Davis RW, Shen P, Cavalli-Sforza LL, Oefner PJ. 1999. Distribution of haplotypes from a chromosome 21 region distinguishes multiple prehistoric human migrations. Proc Natl Acad Sci USA 96:3796-3800.

Jones AC, Austin J, Hansen N, Hoogendoorn B, Oefner PJ, Cheadle JP, O'Donovan MC. 1999. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. Clin Chem 45(8):1133-1140.

Kanaar R, Hoeijmakers JH, van Gent DC. 1998. Molecular mechanisms of DNA double strand break repair. Trends Cell Biol 8:483-489.

Kendall M, Stuart A. 1979. The advanced theory of statistics. Volume 4. New York: Macmillan Publishing Co. pp580-585.

Khanna KK. 2000. Cancer risk and the ATM gene: a continuing debate. J Natl Cancer Inst 92:795-802.

Kharbanda S, Bharti A, Pei D, Wang J, Pandey P, Ren R, Weichselbaum R, Walsh CT, Kufe D. 1996. The stress response to ionizing radiation involves c-Abl-dependent phosphorylation of SHPTP1. Proc Natl Acad Sci USA 93:6898-6901.

Kovalev S, Mateen A, Zaika AI, O'Hea BJ, Moll UM. 2000. Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. Int J Oncol 16:825-831.

Kuklin A, Munson K, Gjerde D, Haefele R, Taylor P. 1997. Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system. Genet Test 1:201-206.

Lange E, Borresen AL, Chen X, Chessa L, Chiplunkar S, Concannon P, Dandekar S, Gerken S, Lange K, Liang T. 1995. Localization of an ataxia-telangiectasia gene to an approximately 500- kb interval on chromosome 11q23.1: linkage analysis of 176 families by an international consortium. Am J Hum Genet 57: 112-119.

Lavin MF. 1999. ATM: the product of the gene mutated in ataxia-telangiectasia. Int J Biochem Cell Biol 31:735-740.

Lee SJ, Dimtchev A, Lavin MF, Dritschilo A, Jung M. 1998. A novel ionizing radiation-induced signaling pathway that activates the transcription factor NF-κB. Oncogene 17:1821-1826.

Li N, Banin S, Ouyang H, Li G, Courtois G, Shiloh Y, Karin M, Rotman G. 2000. ATM is required for IKK activation in response to DNA double strand breaks. J Biol Chem In press.

Liu W, Qian C, Francke U. 1997. Silent mutation induces exon skipping of fibrillin-1 gene in Marfan syndrome. Nat Genet 16:328-329.

Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. 1998. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic Acids Res 26:1396-1400.

Mai M, Yokomizo A, Qian C, Yang P, Tindall DJ, Smith DI, Liu W. 1998. Activation of p73 silent allele in lung cancer. Cancer Res 58:2347-2349.

Maillet P, Vaudan G, Chappuis P, Sappino AP. 1999. PCR-mediated detection of a polymorphism in the ATM gene. Mol Cell Probes 13:67-69.

Martinho R, Lindsay HD, Flaggs G, DeMaggio AJ, Hoekstra MF, Carr AM, Bentley NJ. 1998. Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. EMBO J. 17:7239-7249.

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*. Proc Natl Acad Sci USA 97:10389-10394.

McCallum CM, Comai L, Greene EA, Henikoff S. 2000. Targeted screening for induced mutations. Nat Biotechnol 18:455-457.

Mehta CR, Patel NR. 1983. A network algorithm for performing Fisher's exact test in rxc contingency tables. J Amer Stat Assoc 78:427-434.

Morrison C, Sonoda E, Takao N, Shinohara A, Yamamoto K, Takeda S. 2000. The controlling role of ATM in homologous recombinational repair of DNA damage. EMBO J 19:463-471.

Nickerson ML, Weirich G, Zbar B, Schmidt LS. 2000. Signature-based analysis of MET proto-oncogene mutations using DHPLC. Hum Mutat 16:68-76.

O'Donovan MC, Oefner PJ, Roberts SC, Austin J, Hoogendoorn B, Guy C, Speight G, Upadhyaya M, Sommer SS, McGuffin P. 1998. Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics 52:44-49.

Oefner PJ, Underhill PA. 1998. DNA mutation detection using denaturing high performance liquid chromatography (DHPLC). In: Current protocols in Human Genetics 7.10.1-7.10.12.

Piret B, Schoonbroodt S, Piette J. 1999. The ATM protein is required for sustained activation of NF-kappaB following DNA damage. Oncogene 18:2261-2271.

Richard I, Beckmann JS. 1995. How neutral are synonymous codon mutations? Nat Genet 20:259.

Roest PA, Roberts RG, Sugino S, vanOmmen GJ, denDunnen JT. 1993. Protein truncation test (PTT) for rapid detection of translation- terminating mutations. Hum Mol Genet 2:1719-1721.

Sandoval N, Platzer M, Rosenthal A, Dork T, Bendix R, Skawran B, Stuhrmann M, Wegner RD, Sperling K, Banin S, Shiloh Y, Baumer A, Bernthaler U, Sennefelder H, Brohm M, Weber BHF, Schindler D. 1999. Characterization of ATM gene mutations in 66 ataxia telangiectasia families. Hum Mol Genet 8(1):69-79.

Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT. 1998. Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res 58:4375-4382.

Sasaki T, Tian H, Kukita Y, Inazuka M, Tahira T, Imai T, Yamauchi M, Saito T, Hori T, Hashimoto-Tamaoki T, Komatsu K, Nikaido O, Hayashi K. 1998. ATM

mutations in patients with ataxia-telangiectasia screened by a hierarchical strategy. Hum Mutat 12:186-195.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268:1749-1753.

Schaffner C, Idler I, Stilgenbauer S, Dohner H, Lichter P, 2000. Mantle cell lymphoma is characterized by inactivation of the ATM gene. Proc Natl Acad Sci USA 97:2773-2778.

Schriml LM, Peterson RJ, Gerrard B, Dean M 2000. Use of denaturing HPLC to map human and murine genes and to validate single-nucleotide polymorphisms. Biotechniques 28:740-745.

Sedgwick RP, Boder E. 1991. Ataxia-telangiectasia. In: Vianney De Jong JMB, editor. Hereditary and Spinocerebellar Atrophies. Amsterdam: Elsevier, Inc. pp 347-423.

Shafman T, Khanna KK, Kedar P, Spring K, Kozlov S, Yen T, Hobson K, Gatei M, Zhang N, Watters D, Egerton M, Shiloh Y, Kharbanda S, Kufe D, Lavin MF. 1997. Interaction between ATM protein and c-Abl in response to DNA damage. Nature 387:520-523.

Skopek TR, Glaab WE, Monroe JJ, Kort KL, Schaefer W. 1999. Analysis of sequence alterations in a defined DNA region: comparison of temperature-

modulated heteroduplex analysis and denaturing gradient gel electrophoresis. Mutat Res 430:13-21.

Stankovic T, Weber P, Stewart G, Bedenham T, Murray J, Byrd PJ, Moss PA, Taylor AM. 1999. Inactivation of ataxia telangiectasia mutated gene in B-cell chronic lymphocytic leukaemia. Lancet 353:26-29.

Stilgenbauer S, Schaffner C, Winkler D, Ott G, Leupolt E, Bentz M, Moller P, Muller-Hermelink HK, James MR, Lichter P, Dohner H. 2000. The ATM gene in the pathogenesis of mantle-cell lymphoma. Ann Oncol 11:127-130.

Swift M, Morrell D, Cromartie E, Chamberlin AR, Skolnick MH, Bishop DT. 1986. The incidence and gene frequency of ataxia-telangiectasia in the United States. Am J Hum Genet 39:573-583.

Syllaba K, Henner K. 1926. Contribution a l'independence de l'athetose double idiopathique et congenitale. Atteinte familiale, syndrome dystrophique, signe de reseau vasculaire conjonctival, integrite psychique. Review of Neurology 1:541-562.

Taniguchi K, Krishnadath KK, Qian C, Yang P, Smith DI, Wang KK, Liu W. 2000. High sensitivity of denaturing high performance liquid chromatography (DHPLC) in detection of p53 mutations in adenocarcinoma of the esophagus. Proceedings of the 91st Annual Meeting of the American Association for Cancer Research. 41:624.

Telatar M, Teraoka S, Wang Z, Chun HH, Liang T, Castellvi-Bel S, Udar N, Borresen-Dale AL, Chessa L, Bernatowska-Matuszkiewicz E, Porras O, Watanabe M, Junker A, Concannon P, Gatti RA. 1998. Ataxia-telangiectasia: identification and detection of founder-effect mutations in the ATM gene in ethnic populations. Am J Hum Genet 62:86-97.

Teraoka SN, Telatar M, Becker-Catania S, Liang T, Onenguit S, Tolun A, Chessa L, Sanal O, Bernatowska E, Gatti RA, Concannon P. 1999. Splicing defects in the ataxia-telangiectasia gene, ATM: Underlying mutations and consequences. Am J Hum Genet 64:1617-1631.

Tilanus-Linthorst MM, Obdeijn IM, Bartels KC, de Koning HJ, Oudkerk M. 2000. First experiences in screening women at high risk for breast cancer with MR imaging. Breast Cancer Res Treat 63:53-60.

Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ. 1997. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 7:996-1005.

Vorechovsky I, Rasio D, Luo L, Monaco C, Hammarstrom L, Webster AD, Zaloudik J, Barbanti-Brodani G, James M, Russo G. 1996a. The ATM gene and susceptibility to breast cancer: analysis of 38 breast tumors reveals no evidence for mutation. Cancer Res 56:2726-2732.

Vorechovsky I, Luo L, Lindblom A, Negrini M, Webster DB, Croce CM, Hammarstrom L. 1996b. ATM mutations in cancer families. Cancer Res 56:4130-4133.

Vorechovsky I, Luo L, Dyer MJ, Catovsky D, Amlot PL, Yaxley JC, Foroni L, Hammarstrom L, Webster AD, Yuille MA. 1997. Clustering of missense mutations in the ataxia-telangiectasia gene in a sporadic T-cell leukaemia. Nat Genet 17:96-99.

Wagner TM, Moslinger RA, Muhr D, Langbauer G, Hirtenlehner K, Concin H, Doeller W, Haid A, Lang AH, Mayer P, Ropp E, Kubista E, Amirimani B, Helbich T, Becherer A, Scheiner O, Breiteneder H, Borg A, Devilee P, Oefner P, Zelinski C. 1998. BRCA1-related breast cancer in Austrian breast and ovarian cancer families: specific BRCA1 mutations and pathological characteristics. Int J Cancer 77:354-360.

Wagner T, Stoppa-Lyonnet D, Fleischmann E, Muhr D, Pages S, Sandberg T, Caux V, Moeslinger R, Langbauer G, Borg A, Oefner P. 1999a. Denaturing high-performance liquid chromatography detects reliably BRCA1 and BRCA2 mutations. Genomics 62:369-376.

Wagner TM, Hirtenlehner K, Shen P, Moeslinger R, Muhr D, Fleischmann E, Concin H, Doeller W, Haid A, Lang AH, Mayer P, Petru E, Ropp E, Langbauer G, Kubista E, Scheiner O, Underhill P, Mountain J, Stierer M, Zielinski C, Oefner P. 1999b. Global sequence diversity of BRCA2: analysis of 71 breast cancer families and 95 control individuals of worldwide populations. Hum Mol Genet 8:413-423.

Watters D, Khanna KK, Beamish H, Birrell G, Spring K, Kedar P, Gatei M, Stenzel D, Hobson K, Kozlov S, Zhang N, Farrell A, Ramsay J, Gatti R, Lavin M. 1997. Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. Oncogene 14:1911-1921.

Werneke U. 1997. Ataxia telangiectasia and risk of breast cancer. Lancet 350: 739-740.

Woods CG, Bundey SE, Taylor AMR. 1990. Unusual features in the inheritance of ataxia-telangiectasia. Hum Genetics 84:555-562.

Yokomizo A, Tindall DJ, Drabkin H, Gemmill R, Franklin W, Yang P, Sugio K, Smith DI, Liu W. 1998. PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. Oncogene 17:475-479.

TABLE I. Breast Cancer Patient Characteristics and Tumor Properties

Patient demographics and tumor characteristics	Number of patients
Age group (years)	
Under 40	2
40-59	20
60-80	29
Over 80	1
Ethnic group	
Caucasian[a]	26
Hispanic	18
Black	7
Asian	1
Menopausal status	
Premenopausal	7
Postmenopausal	45
Parent diagnosed with cancer	
Breast	2
Breast and other site	3
Other Site	8
None	39
Stage of disease	
Stage 0	6
Stage I	31
Stage II	14
Stage III	1
Histology	
Ductal carcinoma in-situ	3
Invasive ductal carcinoma	46
Invasive lobular carcinoma	3

[a]9 patients of Ashkenazi Jewish background

Table II. Mutations and Polymorphisms Identified in Breast Cancer Patients Using DHPLC

A Non-Conservative Missense

Patient Number	Location	Exon	Codon Number	Amino Acid Change	Reported/ Novel
30	2119T→C	15	707	ser→pro	Novel
45	4138C→T	30	1380	his→tyr	Novel
45	4400A→G	31	1467	asp→gly	Novel
49	2362A→C	17	788	ser→arg	Novel

B Conservative Missense

Patient Number	Location	Exon	Codon Number	Amino Acid Change	Reported/ Novel
3	5557G→A	39	1853	asp→asn	Reported[abc]
7	4258C→T	31	1420	leu→phe	Reported[de]
8	5557G→A	39	1853	asp→asn	Reported[abc]
18	378T→A	7	126	asp→glu	Reported[fg]
19	2572T→C	19	858	phe→leu	Reported[e]
19	5557G→A	39	1853	asp→asn	Reported[abc]
20	5557G→A	39	1853	asp→asn	Reported[abc]
33	5557G→A	39	1853	asp→asn	Reported[abc]
34	5557G→A	39	1853	asp→asn	Reported[abc]
34	7397C→T	52	2466	ala→val	Novel
41	5557G→A	39	1853	asp→asn	Reported[abc]
42	5557G→A	39	1853	asp→asn	Reported[abc]
43	2572T→C	19	858	phe→leu	Reported[e]
44	5557G→A	39	1853	asp→asn	Reported[abc]
49	6088A→G	43	2030	ile→val	Novel
50	378T→A	7	126	asp→glu	Reported[fg]
50	5557G→A	39	1853	asp→asn	Reported[abc]

C Synonymous

Patient Number	Location	Exon	Codon Number	Amino Acid Change	Reported/ Novel
12	5793T→C	41	1931	ala→ala	Novel
21	1176C→G	11	392	gly→gly	Novel
34	735C→T	9	245	val→val	Reported[h]

D Intronic

Patient Number	Location	Reported/ Novel
1	IVS4+36insAA	Reported[a]
1	IVS17-56G→A[i]	Reported[a]
1	IVS22-77T→C	Reported[ab]
1	IVS48-69ins3	Reported[b]
2	IVS4+36insAA	Reported[a]
2	IVS17-56G→A	Reported[a]
2	IVS22-77T→C	Reported[ab]
2	IVS25-14delA	Reported[a]
2	IVS48-69ins3	Reported[b]
3	IVS4+36insAA	Reported[a]
4	IVS4+36insAA	Reported[a]
4	IVS17-56G→A	Reported[a]
4	IVS25-14delA	Reported[a]
4	IVS48-69ins3	Reported[b]
5	IVS22-77T→C	Reported[ab]
5	IVS48-69ins3	Reported[b]
7	IVS17-56G→A	Reported[a]
7	IVS22-77T→C	Reported[ab]
7	IVS25-14delA	Reported[a]
7	IVS48-69ins3	Reported[b]
8	IVS17-56G→A	Reported[a]
8	IVS48-69ins3	Reported[b]
10	IVS17-56G→A	Reported[a]
10	IVS22-77T→C	Reported[ab]
10	IVS25-14delA	Reported[a]
10	IVS48-69ins3	Reported[b]
12	IVS4+36insAA	Reported[a]
12	IVS16-18insT	Novel
12	IVS20+28insA	Novel
12	IVS25-14delA	Reported[a]
12	IVS48-69ins3	Reported[b]
15	IVS25-14delA	Reported[a]
16	IVS4+36insAA	Reported[a]
16	IVS48-69ins3	Reported[b]
16	IVS17-56G→A	Reported[a]
18	IVS22-77T→C	Reported[ab]
18	IVS33-20A→G	Reported[a]
18	IVS42-125T→G	Novel
19	IVS4+36insAA	Reported[a]
20	IVS4+36insAA	Reported[a]
21	IVS25-14delA	Reported[a]
22	IVS17-56G→A	Reported[a]

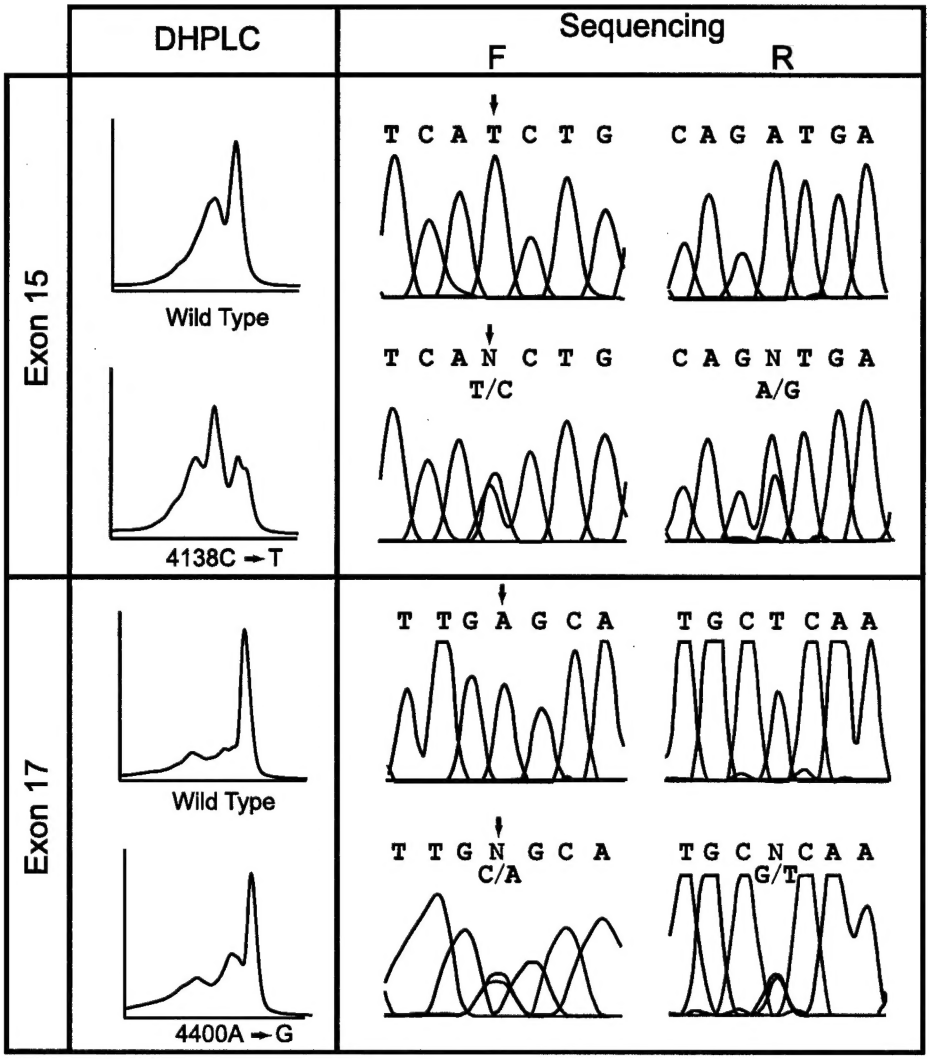
Patient Number	Location	Reported/ Novel
22	IVS22-77T→C	Reported[ab]
22	IVS25-14delA	Reported[a]
22	IVS40+26C→T	Novel
22	IVS48-69ins3	Reported[b]
23	IVS4+36insAA	Reported[a]
23	IVS17-56G→A	Reported[a]
23	IVS22-77T→C	Reported[ab]
23	IVS25-14delA	Reported[a]
23	IVS48-69ins3	Reported[b]
24	IVS4+36insAA	Reported[a]
24	IVS25-14delA	Reported[a]
24	IVS48-69ins3	Reported[b]
26	IVS4+36insAA	Reported[a]
27	IVS4+36insAA	Reported[a]
27	IVS17-56G→A	Reported[a]
27	IVS22-77T→C	Reported[ab]
27	IVS25-14delA	Reported[a]
27	IVS48-69ins3	Reported[b]
28	IVS4+36insAA	Reported[a]
28	IVS17-56G→A	Reported[a]
28	IVS48-69ins3	Reported[b]
29	IVS4+36insAA	Reported[a]
29	IVS17-56G→A	Reported[a]
29	IVS22-77T→C	Reported[ab]
29	IVS25-14delA	Reported[a]
29	IVS48-69ins3	Reported[b]
30	IVS4+36insAA	Reported[a]
30	IVS48-69ins3	Reported[b]
32	IVS4+36insAA	Reported[a]
32	IVS17-56G→A	Reported[a]
32	IVS22-77T→C	Reported[ab]
32	IVS48-69ins3	Reported[b]
33	IVS17-56G→A	Reported[a]
33	IVS25-14delA	Reported[a]
33	IVS48-69ins3	Reported[b]
34	IVS4+36insAA	Reported[a]
34	IVS17-56G→A	Reported[a]
34	IVS22-77T→C	Reported[ab]
34	IVS25-14delA	Reported[a]
34	IVS48-69ins3	Reported[b]
35	IVS4+36insAA	Reported[a]
35	IVS48-69ins3	Reported[b]

Patient Number	Location	Reported/ Novel
37	IVS4+36insAA	Reported[a]
37	IVS25-14delA	Reported[a]
37	IVS48-69ins3	Reported[b]
38	IVS4+36insAA	Reported[a]
38	IVS17-56G→A	Reported[a]
38	IVS19-30del6	Novel
39	IVS22-77T→C	Reported[ab]
40	IVS4+36insAA	Reported[a]
40	IVS17-56G→A	Reported[a]
40	IVS22-77T→C	Reported[ab]
40	IVS48-69ins3	Reported[b]
41	IVS4+36insAA	Reported[a]
42	IVS4+36insAA	Reported[a]
42	IVS14-55T→G	Reported[b]
44	IVS17-56G→A	Reported[a]
44	IVS22-77T→C	Reported[ab]
44	IVS38-15G→C	Novel
44	IVS48-69ins3	Reported[b]
45	IVS4+36insAA	Reported[a]
45	IVS17-56G→A	Reported[a]
45	IVS19-17G→T	Novel
45	IVS40+26C→T	Novel
45	IVS48-69ins3	Reported[b]
46	IVS14-55T→G	Reported[b]
47	IVS4+36insAA	Reported[a]
48	IVS4+36insAA	Reported[a]
48	IVS17-56G→A	Reported[a]
48	IVS22-77T→C	Reported[ab]
48	IVS48-69ins3	Reported[b]
49	IVS4+36insAA	Reported[a]
49	IVS5-17A→G	Novel
49	IVS22-77T→C	Reported[ab]
49	IVS48-69ins3	Reported[b]
50	IVS17-56G→A	Reported[a]
50	IVS33-20A→G	Reported[a]
50	IVS38-8T→C	Reported[ab]
50	IVS48-69ins3	Reported[b]
51	IVS4+36insAA	Reported[a]
51	IVS19-17G→T	Novel
51	IVS48-69ins3	Reported[b]
52	IVS4+36insAA	Reported[a]

[a]Sandoval et al 1999
[b]Castellvi-Bel et al 1999
[c]Maillet et al 1999
[d]Vorechovsky et al 1996a
[e]Vorechovsky et al 1996b
[f]Sasaki et al 1998
[g]Ejima et al 2000
[h]Hacia et al 1998
[i]The location IVS17-56 refers to Intervening Sequence (intron) 17, 56 base pairs from the 3' end of the intron. If the IVS number is followed by a + and a number, it indicates the number of base pairs from the 5' end of the intron.

Figure Legend

Fig. 1. The DHPLC chromatogram and sequencing profiles for the exon 15 and exon 17 PCR products. F designates the forward sequence and R is the confirmatory reverse sequence (reverse compliment of the forward sequence). The arrows indicate the nucleotide in the forward sequence that is mutated in each exon. The double nucleotide band presented in the mutated DNA appears as a double peak and the nucleotides present on each chromosomal copy of the gene are indicated below the N in each case.


DHPLC
Sequencing
F
R
Exon 15
Wild Type
T C A T C T G
C A G A T G A
4138C → T
T C A N C T G
T/C
C A G N T G A
A/G
Exon 17
Wild Type
T T G A G C A
T G C T C A A
4400A → G
T T G N G C A
C/A
T G C N C A A
G/T